In Vitro and *In Situ* Porcine Models for the Studies on Phenotypic Characterization of Cartilage Cells During Endochondral Ossification

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ABSTRACT

The process of endochondral ossification occurring in growth plate cartilage is critical in skeletal development, bone growth and fracture healing. During this process, growth plate chondrocytes differentiate from resting stage through proliferative stage to terminal hypertrophic stage in a highly organized manner. Due to technical difficulties in obtaining pure chondrocytes in different stages of maturation during endochondral ossification, most of the previous investigations on growth plate cells used a mixed population of chondrocytes. We hypothesize that cartilage cells at various differentiation states are different in their metabolism and responses to various hormones and stimulators. Using *in vitro* and *in situ* porcine costal growth plate models established in this study, we successfully identified and separated the various differentiative chondrocyte subpopulations. In addition, their phenotypic characteristics, including basic biochemistry, glycoconjugate expressions, calcium metabolism, electrophysiology and drug responses have been investigated.

Chondrocyte mixture released from growth plate cartilage was applied to countercurrent centrifugal elutriation. Eighteen fractions of chondrocytes with different sizes and densities were obtained. Their mean cellular volumes increased progressively in each of the successive fractions and that increase was accompanied with specific phenotypic changes such as differences in DNA synthesis, proteoglycan synthesis and alkaline phosphatase activities. Three distinct maturational growth plate subpopulations, each with their own unique biochemical characteristics, were identified among the elutriated fractions. The resting chondrocytes were small and quiescent. The hypertrophic chondrocytes were large and metabolically active in alkaline phosphatase as well as proteoglycan production. The proliferative population represents the cells with high DNA synthesis potential and its size range lies between the resting and hypertrophic cells.

Glycosylation of proteins is one of the most important post-translational modifications. In this study, the glycoconjugates of intracellular component and extracellular matrix of growth plate cartilage *in situ* were analyzed by lectin histochemistry. The N-acetylgalactosamine and galactose containing glycoconjugates in the matrix became increasingly abundant from resting zone to the hypertrophic zone. Moreover, β-galactose was commonly expressed in growth plate chondrocytes, since chondrocytes in all zones were positively labeled with peanut agglutinin (PNA), *Ricinus communis* agglutinin-I (RCA-I) and RCA-II. Therefore, the RCA-I binding glycoconjugates demonstrated in the growth plate cartilage in this study may represent the fibronectin. Succinyl-*Canavalia ensiformis* agglutinin (S-Con A), a α-mannose binding lectin, bound to the membranes of proliferative and hypertrophic chondrocytes. The cytoplasm of chondrocytes at all zones were also stained. These findings suggest that α-mannose may be essential to growth plate chondrocytes. The intracellular binding with S-Con A in the chondrocytes probably represents stored glycogen.

The results of the lectin histochemistry study showed that the differentiating chondrocytes in the porcine physeal growth plate express characteristic lectin binding patterns. In the physeal growth plate, wheat germ agglutinin (WGA) bound specifically to the resting chondrocytes, while *Dolichos biflous* agglutinin (DBA) and *Phaselous vulgaris* agglutinin-E (PHA-E) bound to the proliferative and hypertrophic zone chondrocytes. These lectins can be used as the differentiation markers for the growth plate chondrocytes.

The physiologic significance of large amounts of intracellular free calcium $[Ca^{2+}]_i$ in the growth plate chondrocytes has not yet been determined. But it has been postulated to play a role in matrix mineralization and regulation of cellular metabolism. Since attachment of cartilage matrix is critical for maintaining the physiological behavior of calcium metabolism in chondrocytes, a new model using longitudinal growth plate slices for *in situ* monitoring of intracellular and

extracellular Ca^{2+} with laser scanning confocal microscopy from each zone of the porcine growth plate has been established in this study.

From the results of this study, there appears a general trend for the intracellular free calcium to concentrate in the nuclei of the resting chondrocytes through the proliferative chondrocytes to the sub-plasma membrane region in the hypertrophic chondrocytes. This indicates that in the hypertrophic chondrocytes and proliferative chondrocytes of maturation zone, free calcium is mainly trapped inside the Golgi apparatus and endoplasmic reticulumn for cellular secretion through matrix vesicles. This finding has been supported by evidence that free calcium arcs and islands were detected in the hypertrophic chondrocytes and proliferative chondrocytes respectively.

Moreover, there are differential responses on intracellular calcium in growth plate chondrocytes among various zones of growth plate to transforming growth factor β_1 (TGF-β_1). In resting chondrocytes, all cells showed a significant increase in calcium level after adding of TGF-β_1. In proliferative chondrocytes, some cells were responsive to TGF-β_1 while others were not. For the responsive cells, the stimulation of intracellular calcium was much more mild when compared with resting chondrocytes. There was no response on intracellular calcium to TGF-β_1 in the hypertrophic chondrocytes. In this study, triggering of intracellular Ca^{2+} increase by the binding of TGF-α to the growth plate chondrocytes for all of the three maturation stages has been demonstrated. It is a well established fact that parathyroid hormone (PTH) regulates the calcium level in plasma and bone. However, in the porcine costal growth plate chondrocyte model established in this study, the effect of PTH on chondrocytes does not seem to involve Ca^{2+} as a secondary messenger.

In order to explore the electrophysiological properties of growth plate chondrocytes, an *in situ* model of partially digested growth plate slices for further characterization of the different types of ion channel in various differentiation stages of growth plate chondrocytes has been established . Two types of K^+-channel have been found on the

chondrocyte membranes and subsequently identified. We demonstrated that the major outward current found in the chondrocytes is the delayed K^+ current. In addition, for the first time we were able to show that this outward current is present not only in the resting cells but also expressed in the proliferative and hypertrophic cells. In fact, the latter two types of cells expressed a much higher magnitude of current. This may indicate that such type of channel plays an active role during the process of differentiation. The presence of another calcium-dependent potassium current has been identified in chondrocytes. This current is found to be more sensitive to tetraethylammonia chloride (TEA) when compared with the delayed rectifier.

The differential effects of quinolones, a clinically useful broad spectrum antibiotics, which is known to have side effect of inducing cartilage damage, in different chondrocyte subpopulations were studied. From that results, it appears that quinolones such as ciproxin, ofloxacin, norflorcin and nalidixic acid, affect cell viability of the resting chondrocytes much more seriously than the proliferative and hypertrophic chondrocytes released from growth plate. Our findings indicate that the cell viabilities obtained after treatment for resting and growth plate cells were 83% and 93% respectively. Among four quinolones tested in this study, ofloxacin appears to be the most potent cytotoxic drug for resting chondrocytes but is the least potent one for growth plate chondrocytes.

All quinolones (except ofloxacin) tested in this study showed a general inhibition on thymidine uptake in both resting and growth plate chondrocytes at higher concentrations. The effect appears to be more potent in resting chondrocytes than growth plate chondrocytes. At the concentration of 100 μg/ml, the maximum inhibition on thymidine uptake was 94% in resting chondrocytes while it was only 57% in growth plate chondrocytes. For proteoglycan synthesis activities, although nalidixic acid showed no effect on both resting and growth plate chondrocytes mixture, the effect of other quinolones were irregular in resting when compared with growth plate chondrocytes. In growth plate chondrocytes, all other quinolones

performed a suppression effect on sulfate incorporation. Norflorcin is the most potent inhibitor. All quinolones tested in this study could suppress activity of alkaline phosphatase, a chondrocyte differentiation marker, in growth plate chondrocytes. It indicates that quinolones may inhibit chondrocyte differentiation and in consequence hinder the development of growth plate and then suppress the bone growth.

The pathological effects of quinolones on resting zone and growing zone cartilage are different. In resting chondrocytes, the primary effect is direct cell killing. In the growth plate chondrocyte mixture which contains resting, proliferative and hypertrophic chondrocytes, the major pathology of quinolones is the prevention of chondrocyte differentiation. These observations provide us with further supportive evidence for the hypothesis that porcine chondrocytes at various maturation stages have different phenotypic characteristics and respond differently in the presence of different modulators.

In conclusion, by using *in vitro* and *in situ* porcine costal growth plate models established in this study, we have successfully identified and separated various differentiative chondrocyte subpopulations. In addition, their phenotypic characteristics, such as basic biochemistry, glycoconjugate expression, calcium metabolism, electrophysiology and drug responses have been elucidated. These findings confirmed our hypothesis that cartilage cells at various differentiation stages have different metabolic systems. The cells also respond differently to various hormones and stimulators. Therefore, the maturation stages should be clearly defined in all future investigations in growth plate chondrocytes and the period of using a mixed population of chondrocytes is no longer justified.

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ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular ionized calcium concentration
$[Ca^{2+}]_o$	Extracellular calcium concentration
4AP	4-Aminopyridine
ACP	Amorphous calcium phosphate
ALP	Alkaline Phosphatase
BPA	*Bauhinia purpurea* agglutinin
cAMP	Intracellular cyclic adenosine 3',5'-monophosphate
CCE	Countercurrent centrifugal elutriation
CCK	Cholecystokinin
Con A	*Conavalia ensiformis* agglutinin
CTX	Charybdotoxin
DAG	1,2-diacylglycerol
DAT	Digital audio tape
DBA	*Dolichos biflorus* agglutinin
DMEM	Dulbecco's Modified Eagle's Medium
DSA	*Datura stramonium* agglutinin
ECA	*Erythrina cristagalli* agglutinin
ECF	Extracellular fluid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
E_{rev}	Reversal potential
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Fluo-3/AM	Fluo-3 acetoxy(methyl) ester
Fuc	Fucose
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetyglucosamine

GNA	*Calanthus nivails* agglutinin
GS	*Griffonia simplicifolia* agglutinin
GS-IB	*Griffonia simplicifolia* agglutinin
HAA	*Helix aspersa* agglutinin
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HPA	*Helix pomatia* agglutinin
ICF	Intracellular fluid
I_K	delayed rectified potassium current
IL-1	Interleukin-1
IP_3	Inositol 1,4,5-triphosphate
LCA	*Lens culinaris* agglutinin
LFA	*Limax flavus* agglutinin
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
Lotus A	*Lotus tetragonolobus* agglutinin
LPA	*Limulus polyphemus* agglutinin
MAA	*Maackia amurensis* agglutinin
Man	Mannose
MPA	*Maclura pomifera* agglutinin
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
PHA	*Phaselous vulgaris* agglutinin
Pi	Inorganic phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PK_{Ca}	High conductance Ca^{2+}-activated K^+
PLC	Plasma-membrane enzyme phospholipase C
PM	Plasma membrane
PMT	Photomultiplier tube
PNA	Peanut agglutinin
PSA	*Pisum sativum* agglutinin
PTH	Parathyroid hormone

PWA	Pokeweed agglutinin
RCA	*Ricinus communis* agglutinin
RVD	Regulatory volume decrease
RYR	Ryanodine receptor
SBA	Soy bean agglutinin
SJA	*Sophora japonica* agglutinin
SNA	*Sambucus nigra* agglutinin
STA	*Solanum tuberosum* agglutinin
TEA	Tetraethylammonia
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor alpha
UDA	*Urica diolca* agglutinin
UEA	*Ulex europaeus* agglutinin
UEA	*Ulex europaeus* agglutinin
VVA	*Vicia villosa* agglutinin
WFA	*Wistaria floribunda* agglutinin
WGA	Wheat germ agglutinin

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Chapter One

General Introduction

Cartilage is a specialized dense connective tissue. It is avascular with no nervous tissue present and made up of chondrocytes embedded in a matrix which consists largely of water, collagen fibrils and proteoglycan macromolecules. These macromolecules form a fiber-reinforced gel. The major collagen in cartilage is type II whereas type I collagen is predominant in bone. The characteristics of cartilage are determined by the different amounts of proteoglycan and the ratio of the different types of collagen in the matrix. As all the matrix substances are secreted by the cartilage producing cells, the chondrocytes, it is theoretically possible to produce any types of cartilage by stimulating the chondrocytes to synthesize specific matrix.

Cartilage acts as a temporary skeleton in the embryo and serves as a basic structure on which bones develop. During the growth of new-born mammals, including human, the embryonic cartilaginous skeleton is eventually replaced by bone in a process called endochondral ossification occurring in the growth plate. This process also occurs during bone fracture healing.

The sequence of morphological events involved in the ossification of the distal femoral physeal growth plate of CD1 mouse has been described (Floyd et al., 1987). The epiphysis in the newborn animal is found to be entirely cartilaginous, containing only resting chondrocytes surrounded by matrix. On day two after birth, the chondrocytes at a specific region start to perform uni-directional cell divisions. Thus, columns of proliferative chondrocytes stack up in a parallel manner. On the fifth day, the proliferating chondrocytes differentiate into hypertrophic chondrocytes, which are 5 - 10 times larger in volume than the resting cells. Then capillary invasion and matrix mineralization begin to take place. Finally, osteoblasts carried by the blood deposit bone matrix on the spicules of calcified cartilage. This completes the process of endochondral ossification which is responsible for bone growth. Although the morphological description of resting, proliferative and hypertrophic growth plate chondrocytes has been established for many years, the identification and separation of these chondrocyte subpopulations were not successful in the past.

During the last decades, many investigators have attempted to prepare pure samples of chondrocytes from different zones of growth-plate cartilage by microdissection (Wuthier, 1969; Kuhlman, 1965). However, this method presents problems in reproducibility and requires tedious procedures. Another technique, micropuncture, allows precise localization of the cells to be analyzed, but the number of cells for analysis is limited. Moreover, Ray et al. (1982) reported that Ficoll density gradient could not successfully separate chondrocytes with different cell densities.

Most of the previous investigations on the growth plate chondrocytes are thus done on a population of mixed stages of chondrocytes. We hypothesize that cartilage cells at different differentiation stages have different metabolic systems and respond differently to various hormones and stimulators. The objectives of this study is to identify and separate various differentiative chondrocyte subpopulations for further phenotypic characterizations on their basic biochemistry, glycoconjugate expression, calcium metabolism, electrophysiology and drug responses. The pig, a mammal with similar skeletal development to the human, is chosen as the experimental model for this study.

Although the process of endochondral ossification which occurs in growth plate cartilage is so critical, progress on the study chondrocyte differentiation during the past decades is still very limited. One major problem is that the chondrocytes are being trapped within the rigid cartilage matrix which consists of collagen fibrils and proteoglycan macromolecules. Cell separation cannot be achieved by simple trypsinization, and therefore complicated enzyme digestion procedure with trypsin, hylaruonidase and collagenase is required. Since the matrix compositions vary in different sites in different animals and overdigestion will kill the chondrocytes, it is tedious to optimize enzyme digestion procedure for a specific model.

The maturation process of chondrocyte, from resting stage through proliferative stage to terminal hypertrophic stage, in growth plate progresses in an highly organized manner. The cells released from the growth plate cartilage usually consist of a

mixture of chondrocytes at different maturation stages. Alkaline phosphatase is a well established marker of the hypertrophic chondrocytes and it can be used for the identification of such cell subpopulation but not their separation.

The isolation of chondrocyte subpopulations has become a much active topic in growth plate chondrocytes studies over the last ten years. It was not until 1989 that O'Keefe and colleagues successfully applied a technique called countercurrent centrifugal elutriation (CCE) to separate and identify the chondrocytes from the chick growth plate according to the differences in their cellular sizes and densities. Thereafter, the study of cell biology and biochemistry of the individual chondrocyte subpopulations become possible.

Rosier and colleagues (1989) have found that transforming growth factor-β exhibits a larger mitogenicity on hypertrophic cells and proliferating cells than on smaller resting cells. The same group also reported that type X collagen synthesis was increased in hypertrophic chondrocytes (O'Keefe et al., 1990, Gunter et al., 1990). Their findings clearly show that chondrocytes at various stages of differentiation have their own phenotypic characteristics and respond distinctly to different hormones and modulators. Moreover, these results challenged the pervious investigations using a mixture of growth plate chondrocytes in different maturation stages.

So far, countercurrent centrifugal elutriation has only been applied in chick growth plate but not on other larger mammals of which the skeletal development is closer to human. In this study, CCE was applied to separate porcine costal growth plate chondrocyte subpopulations on the basis of sizes and densities. The alkaline phosphatase activity, the rates of DNA synthesis and proteoglycan synthesis of these cells at various stages of differentiation have also been studied (Chapter 2).

Glycosylation of proteins is one of the most important post-translational modifications preceding or concomitant with their incorporation into various cytoplasmic compartments as well as organelles and their functional activation or secretion (Barondes, 1984; Hakomori, 1985). In contrast to other forms of post-translational modifications such as sulfation or phosphorylation, glycosylation is usually quite complex and may involve sequential action of multiple enzymes leading to the formation of highly heterogeneous glycoproteins. Glycosylation determines many of the final structural and functional properties of proteins, as well as confers to them their essential biological attributes, and directs their turnover and transcellular traffic (Hakomori,1985; Yogeeswaran, 1983). The regulation of glycosylation is of importance for interaction between cells and their environment during development and growth (Stanley, 1987).

The main objective of the study in Chapter 3 is to localize and partially characterize the glycoconjugates expressed by the different subpopulations of chondrocytes in the porcine growth plate by lectin histochemistry. Lectins are specific sugar binding proteins of non-immune origin. A panel of lectins with different sugar binding specificities were used. The information on the differential expression of glycoconjugates in the growth plate chondrocytes obtained in this study can provide deeper insights on the separation and identification of chondrocyte subpopulations in various maturation stages. This can be achieved, according to their different expression of membrane glycoconjugates, by using various methods such as lectin affinity chromatography and flow cytometry with fluorescein-labeled lectin probes. The isolated chondrocyte subpopulations will be further characterized as part of the future project. The findings will provide a better understanding of the process of endochondral ossification and normal bone development.

The cytosolic ionized calcium (Ca^{2+}) concentration has been demonstrated in many cells to play a critical role in the regulation of cellular metabolism. In the growth plate chondrocytes, the content of intracellular free calcium appears to increase during the processes of cellular maturation (from resting stage, through proliferative stage to hypertrophic stage) and matrix mineralization (Iannotti & Brighton, 1989). The physiological significance of the accumulation of large amount of intracellular free calcium , $[Ca^{2+}]_i$, in growth plate chondrocytes has not been determined but has been postulated to play a role in matrix mineralization and regulation of cellular metabolism. Since attachment of cartilage matrix is critical for maintaining the physiological behavior of calcium metabolism in chondrocytes, a new model for *in situ* monitoring of intracellular and extracellular Ca^{2+} of the chondrocyte subpopulations with laser scanning confocal microscopy from each zone of the pig rib growth plate is established (Chapter 3). The development of such *in situ* Ca^{2+} monitoring model enables us to investigate the effect of various modulators, such as TGF-α, TGF-β and PTH on the $[Ca^{2+}]_i$ among chondrocytes at different maturational stages during endochondral ossification.

Although the transmission of signals mediated by ion channels within and between cells is critical for cell differentiation, little is known on the electrophysiological properties of chondrocytes until Grandolfo and his co-workers first investigated the potassium channels using the patch clamp technique in 1990. Among the limited investigations on chondrocyte ion channels, all the studies were in cell culture model. It was well known that differentiation and de-differentiation of chondrocytes under culture condition is unavoidable. This is the reason for the biochemical characterization of elutriated chondrocyte subpopulations to be carried out within 24 hour of cell harvested from CCE in this study. Moreover, since the CCE fractions are only highly enriched with certain chondrocyte subpopulation, a small amount of cross contamination by other subpopulations cannot be eliminated. This would create problems for some individual cell physiology assessments. To avoid this problem, an modification of the above *in situ* growth plate model with partially digested growth

plate slices for further characterization of the existence and properties of various types of ion channels in growth plate chondrocytes has been established (Chapter 5).

In order to apply the established chondrocyte model to clinical research, the differential effects of quinolones, a type of clinically useful broad spectrum antibiotics with evidences of inducing cartilage damage, in different chondrocyte subpopulations were also investigated in this study (Chapter 6). This part of the study was initiated by the worries of the clinical users on quinolones while at the same time was designed as an alternative approach to the study of chondrocyte differentiation which was subjected to adverse effects of harmful antibiotics.

Skeletal development, fracture-healing and joint degeneration, the three main fields in orthopedics research, are directly related to chondrocyte differentiation, especially endochondral ossification. In patients suffering from metatropic dwarfism, the maturation of proliferative chondrocytes into hypertrophic cells was either completely aborted or profoundly incomplete (Boden et al. 1987). Damage to the growth plate is permanent and irreversible. Children so affected, either as a result of trauma or infection, have severe and permanent joint deformities. The affected limb is short and deformed due to the formation of bony bridge in the site of injured area after focal damage of the growth plate. The remaining epiphysis is not allowed to grow and the skeletal development of the affected bone is blocked. A study on the biochemical and electrophysiological characteristics of chondrocyte differentiation, which in turn would contribute to the understanding of the process and control of bone development and fracture-healing is very meaning clinically. Knowledge on the process of progressive cartilage differentiation, would logically lead to more insights about the regressive (degenerative) processes. This is very important in the research of degenerative joint diseases such as osteoarthritis. A better understanding of the chondrocyte differentiation process is therefore essential for the improvement in the treatment of fractures healing, cartilage regeneration and other orthopaedic diseases, such as metatropic dwarfism.

Chapter Two

Identification and Characterization
of
Various Differentiative Growth Plate Chondrocytes
by
Countercurrent Centrifugal Elutriation

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2.1 AIMS OF STUDY

Cartilage is a specialized dense connective tissue which is avascular and does not contain nervous tissue. It is made up of chondrocytes embedded in an extracellular matrix which consists largely of water, collagen fibrils and proteoglycan macromolecules. These macromolecules form a fiber-reinforced gel which contributes to its tensile stress withstanding properties. In all mammals, cartilage acts as temporary skeleton in the embryo and serves as a basic structure on which bones develop. In human adults, the main function of cartilage is to provide support for soft tissues and gliding areas for joints. Moreover, cartilage is also formed during the process of fracture healing.

The characteristics of cartilage are determined by the different amounts of proteoglycan and the ratio of the different types of collagen in the matrix. As all the matrix substances are secreted by the chondrocytes, it is of value to establish an *in vitro* model to facilitate the study of the biochemistry of these cells. A better understanding in the differentiation process of the chondrocytes in the cartilage is essential for the improvement in the treatment of fractures and other orthopaedic diseases.

During the last decade, most of the research works on cartilage have been focused on the composition of the extracellular matrix and the molecular structure of its components with little advance being made in the area of characterization of chondrocytes (reviewed by Delbruck & Gurr, 1986) . One of the reasons may be due to the difficulties in isolating chondrocytes from the cartilage. Most investigators have attempted to prepare pure samples of the different zones of growth-plate cartilage by microdissection. For example, Wuthier (1969) reported the method of scraping off sequential layers of calf growth plate. Kuhlman (1965) reported the

method of taking microtome sections of dog and rabbit growth plates. However, scraping technique presents problems in reproducibility and identification of layers, while microtome method requires tedious procedures including preliminary freezing. Another technique, micropuncture, allows precise localization of the cells to be analyzed, but the potential number of analyses that can be performed is limited. Moreover, Ray et al. (1982) reported that Ficoll density gradient which is a commonly used method in hematological studies cannot be used to separate chondrocytes with different cell densities. Recently, O'Keefe et al. (1989) successfully applied a relative new technique of countercurrent centrifugal elutriation (CCE) to separate and identify the chondrocytes from chick growth plate according to their sizes and densities. When the biochemistry of these cells were studied, the transforming growth factor-β exhibited a larger mitogenicity on hypertrophic cells and proliferating cells than on smaller resting cells (Rosier et al., 1989). It has also been shown that the alkaline phosphatase activity and the type X collagen synthesis are increased in hypertrophic chondrocytes (O'Keefe et al., 1990, Gunter et al., 1990). Therefore it is of importance to investigate whether maturationally distinct fractions of chondrocytes from species other than chick can also be separated by CCE so that the biochemistry of those cells can be elucidated. In this study, we have applied CCE to separate porcine chondrocytes on the basis of sizes and densities. We also studied the alkaline phosphatase activity, the rates of DNA synthesis and proteoglycan synthesis of these cells at various stages of differentiation.

2.2 LITERATURE REVIEW

2.2.1 Structure of Cartilage

Characteristics of Cartilage

Cartilage is a specialized dense connective tissue consisting of cells, and an extracellular collagen fiber network embedded in a hydrophilic matrix composed chiefly of chondroitin sulfate (Turek, 1984). The matrix, which is synthesized and secreted by chondrocytes, contains type II collagen and chondroitin sulfate proteoglycan, which gives cartilage its characteristic stiffness and resiliency. Due to the fact that cartilage is an avascular tissue, the chondrocytes receive only oxygen and nutrients both of which can diffuse readily through the matrix. *In vivo*, chondrocytes reside in small lacunas within the cartilage matrix, where they tend to have a round shape.

Types of Cartilage

There exists three varieties of cartilage *in vivo*, namely hyaline cartilage, fibrocartilage and elastic cartilage. They are distinguishable from each other on the basis of the amount of extracellular matrix and the relative proportions of elastic and collagen fibers embedded in it. In hyaline cartilage, the matrix appears to be homogeneous and consists principally of collagen fibers arranged in a loose meshwork. This type of cartilage is the most common variety and is found on the ventral surface of ribs, on the joint surfaces of bones, and in the tracheal rings. Like hyaline cartilage, the matrix of fibrocartilage consists largely of collagen fibers, but these fibers form many thick bundles. This type of tissue, which can be viewed as a transitional form between cartilage and dense connective tissue, is found at the site of attachments of tendons to bones, where it gradually merges into the dense connective

tissue of the tendons. Elastic cartilage, on the other hand, differs from the other types in that it contains many elastic fibers in its matrix. It occurs in the external ear, Eustachian tube, and epiglottis.

Matrix

Cartilage matrix consisting largely of water, collagen fibrils and proteoglycans (protein-carbohydrate complexes), forms a fiber-reinforced gel. A minute fraction of the matrix lies within the lacuna which contains finely- textured pericellular matrix distinct from the coarsely-fibrous matrix elsewhere. The physico-chemical properties of the matrix and its constituents determine the mechanical properties of cartilage.

Collagen

On average, 50% of the dry weight of cartilage is collagen although the proportion of collagen to other constituents decreases with age. The precursor molecules of the collagen fibrils in cartilage are synthesized and secreted by the chondrocytes but fibrogenesis occurs extracellularly. Collagen fibrils are cross-linked aggregates of tropocollagen molecules with a high tensile strength (15-30 kg/mm^{-2}) which by weight for weight is equivalent to mild steel. Each tropocollagen molecule, 300nm long and 1.5 nm wide, contains three peptide α-chains. Each α-chain is coiled in a left-handed helix and the three chains are twisted together in a right-handed helix (like a rope). The tropocollagen molecules aggregate in bundles to form fibrils of various widths. Strength and stability is conferred on the fibrils by hydrogen bonding and covalent cross-linkages.

In hyaline cartilage, most of the collagen is of the characteristic type II which containing three identical α_1II chains. Cartilage with Type II collagen may possess

enhanced resistance to tear and may also be the only adequate substitute material for damaged articular cartilage.

Type IX collagen is present in carilage and was the the first link protein dicovered between the fibrils and other matrix components. The is composed of three chanins: α1(IX), α2(IX), and α3(IX), and consists of three triple-helical domains and four noncollagenous donains. (Van der Rest et al., 1985; van der Res and Mayne, 1987). An interesting feature of type IX collagen is tis relationship to proteoglycans. After isolation of a collagenase-senitive proteoglycan from chick embryo physeal cartilage by Noro et al. (1983), it was found to be identical with type IX collagen. Further studies from various groups revealed that the α2(IX) chain is bound to one or two molecules of chondroitin sulfate (Bruchkner et al. 1985; Vaughan et al. 1988). The precise binding site for the glycosaminoglycan side chain is unknown. Type IX collagen is unable to form fibrils. The molecule is highly flexible. It has been suggested that type IX collagen is involved in organizing or maintaining the network of collagen fibrils in cartilge (Eyre et al. 1987; Wotton et al. 1988).

Type XI collagen, known also as 1α2α3α collagen, was discovered as another minor collagen of cartilge (Burgeson and Hollister, 1979). It forms small fibrils. Type XI collagen was isolated rom various hyalie cartilages, including physeal growth cartilage and growth plate as well as articular, costosternal cartilage (Hartmann et al., 1983; Mayne, 1989). It is closed associated with types II and V collagens

In vitro studies of chick embryo morphogenesis showed that chondrocytes at various developmental stages synthesize different types of collagens (Floyd et al, 1987). The committed mesenchymal cells (i.e. precursor of chondrocytes), firstly found in the limb bud and then in the perichondrium, produce type I collagen and fibronectin and possibly minimal amount of type II collagen. Proliferating chondrocytes produce large amounts of type II collagen and cartilage-specific proteoglycans. The

hypertrophic chondrocytes localized in the hypertrophic calcifying cartilage region are characterized by their ability to synthesize type X in addition to type II collagen.

Proteoglycan

Proteoglycans are large hydrophilic and negatively charged macromolecules (mol. wt 1-2 x 10^6 daltons) consisting of a linear protein core to which are attached 50-100 side chains of glycosaminoglycans, chondroitin sulfate and keratan sulfate. The side chains of this components are linear carbohydrate polymers with large numbers of carboxyl and sulfate groups. This highly fixed negative charges attract large number of positively charged counter-ions (such as Ca^{2+})and results in a high osmolarity, which largely accounts for the retention of water in cartilage. The predominant glycosaminoglycan is chondroitin sulfate, which consists of a repeating disaccharide unit of N-acetyl galactosamine and glucuronic acid. Each side chain, on average, contains 25-30 repeating units. Keratan sulfate consists of a repeating disaccharide unit of N-acetyl glucosamine and galactose, and the chains are shorter (about 13 repeating units) than in chondroitin sulfate. Two isomeric forms of chondroitin sulfate occur, chondroitin 4-sulfate and chondroitin 6-sulfate, but the latter predominates in adult cartilage. However, the position of sulfate groups and the degree of sulfatation can vary along the chain. The significance of the position of the sulfate groups remains to be determined. In chondroitin 6- sulfate, the sulfate groups project further from the chain than that of in chondroitin 5-sulfate and may therefore interact with collagen and other proteins to a greater extent.

In cartilage, the majority of the proteoglycans form aggregates with hyaluronic acid, an non-sulfated glycosaminoglycan of 102 μm long. About 30-50 proteoglycans attach, at intervals of 25-50 nm, along the hyaluronic acid molecule. The proteoglycan-hyaluronate aggregate is very large (molecular weight approximately 50 x 10^6 daltons) and is about 1 μm diameter when fully hydrated. In normal

cartilage the aggregates are compressed to about one fifth of their potential volume and have a tendency to swell.

It is important to note that, in articular cartilage, the content of proteoglycan varies from joint to joint and among individuals. An increase in the proteoglycan content is observed from the surface to deeper layers of the articular cartilage and the distribution of different glycosaminoglycans also varies in relationship to depth and with proximity to the cells. In addition, an increase in the proportions of chondroitin 6-sulfate occurs with age, as does that of keratan sulfate has been reported (Mankin and Lippiello, 1970).

Diffusion of Solutes in Cartilage

The proteoglycans and collagen content of the matrix regulate passage of solutes into and through cartilage. Firstly, since diffusion is most rapid in water, solutes must pass more slowly through cartilage, which is never more than 90% water. Secondly, the macromolecules obstruct the passage of solutes, by imposing an increasingly restricted and tortuous pathway of diffusion with increasing size of the solute and by imparting a frictional retardation on the velocity of the solute. Thirdly, the negative charges on the proteoglycans attract cations but repel anions and the magnitude of this effect depends also on the tonicity of the external solution.

Thus, uncharged nutrient molecules and metabolites of small molecular size, such as glucose, diffuse into and through cartilage comparatively easily. Large molecules, such as the γ-globulins, are almost completely excluded from cartilage; although very long and narrow molecules, such as hyaluronic acid, may diffuse with anomalous rapidity through the tissue. Anionic metabolites such as sulfate can diffuse into cartilage, although less easily than cations such as calcium.

Chondrocytes

The size of chondrocyte varies from about 10 μm diameter in articular cartilage to about 30 μm diameter in the hypertrophic zone of the growth plate. Although chondrocytes often lie in groups, cell to cell contacts among chondrocytes rarely if ever, occur in adult cartilage. The chondrocyte surface has the usual antigens and receptors but it is shielded from cytotoxic antibodies by the matrix, which is just barely permeable to high molecular weight proteins. The cells lie in spheroidal lacunae, although the cell itself has a scalloped contour with many projecting cell processes about 1-2 μm long. The tips of the constantly moving processes may become detached to form vesicles in the matrix near the lacunas rim. As in many specialized cells, the chondrocyte nuclei tend to be small and densely basophilic. Aging nuclei often assume bizarre irregular and lobulated shapes.

During embryonic development, cartilage differentiates from mesenchyme, and the mesenchyme is usually of mesodermal origin. However, mesenchyme may also develop from ectoderm, as in the cartilage derived from cranial neural crest (mesectoderm). Chondrogenic mesenchyme from the above mentioned source can undergo chondrogenesis in tissue culture.

It is generally accepted that chondrocytes of adult articular cartilage do not undergo mitotic division. This can be shown by uptake experiment using tritiated thymidine. That uptake could not be detected in mature articular cartilage, whereas immature cartilage showed two zones of division. The first zone was in the superficial layer whereas the second was near the osseous center. It has been suggested that the division occur in the superficial zone contributed to growth of the articular surface and that of the deep zone contributed to the ossification center.

Nevertheless, cell division has been demonstrated in adult cartilage when the surface has become damaged either in early osteoarthritis or chondromalacia. Uptake of tritiated thymidine by cell clusters of chondrocytes has also been shown under experimental injury conditions.

In summary, the cartilage matrix, which is synthesized and secreted by chondrocytes, is highly complex in nature. There is constant interaction between the cells and matrix. Due to the rigid nature of the matrix, one can easily see the difficulties in isolating chondrocytes from cartilage. This is one of the reasons why chondrocyte research has been relatively slow in the past few decades.

2.2.2 Endochondral Ossification

A major function of cartilage is to serve as a structural component of the embryonic skeleton. The cartilage matrix, which is resilient enough to permit cell growth yet strong enough to provide support, makes the tissue ideally suited for this function.

In the central region of embryonic cartilaginous center, the chondrocytes enlarge and become arranged radically. Lime salts are deposited in the matrix. This calcified cartilage disintegrates and is destroyed by invoking vascular tissue from the perichondrium. At the same time the invasive bud-like mass differentiates into osteoblasts thus initiating bone formation. This spongy bone formation continues to replace the cartilage, by extending proximally and distally, resulting in longitudinal growth of the bone. Most of the embryonic cartilaginous skeleton is eventually replaced by bone and marrow in such a process called endochondral ossification, but some cartilage persists into adult life.

The endochondral ossification center can be divided into 5 zones (Jungueir and Carneire, 1980): (1) The **resting zone** consisting of hyaline cartilage without morphological changes in the homogeneous cells. (2) In the **proliferative zone**, chondrocytes divide rapidly and form parallel rows of stacked cells along the long axis of the bone. (3) The **hypertrophic cartilage zone** containing large chondrocytes whose cytoplasm has accumulated glycogen. The resorbed matrix is reduced to thin septa between the chondrocytes. (4) In the **calcified cartilage zone**, the thin septa of cartilage matrix become calcified by the deposition of hydroxyapatite. (5) In the **ossification zone**, endochondral bone tissue appears. Blood capillaries and undifferentiated cells formed by mitosis of cells originating from the periosteum invade the cavities left by the chondrocytes. The undifferentiated cells developed into osteoblasts, which in turn form a discontinuous layer over the septa of calcified cartilage matrix. Over these septa, the osteoblasts lay down bone matrix. During endochondral ossification, chondrocytes undergo a programmed sequence of biochemical and morphological changes. In maturing cartilage, zones of chondrocytes can be seen at various stages in sequence, with progressive stages marked by changes in shapes from round to flattened to hypertrophic. Such morphological changes provide us the physical parameters for identifying chondrocytes in different stages of maturity. Rooney et al. (1984) have suggested that these shape changes may be influenced by the surrounding matrix. Shinomura et al. (1984) and Kimata et al., (1984) have reported the appearance of a specific proteoglycan (PG- Lb) as the maturing chondrocytes acquire a flattened shape. It is not clear, however, whether the change in the shape of chondrocytes is related to expression of the new molecule or not. On the other hand, hypertrophic chondrocytes produce type X collagen and this specific type of collagen is being accepted as the marker for hypertrophic cartilage (Schmid and Linsemayer, 1985). Interestingly, chondrocytes derived from cartilage that normally does not undergo

hypertrophy can be induced to synthesize type X collagen *in vitro* (Solurshet et al., 1986).

Recently, the sequence of events which leads to the formation of the ossification center in the distal femoral epiphysis of the CD1 mouse has been demonstrated by Floyed et al. (1987). The distal femoral epiphysis in the newborn mouse was found to consist entirely of cartilage, containing no vessels and no hypertrophic chondrocytes. An island of hypertrophic chondrocytes appeared in five day old CD1 mouse. On the sixth day of neonatal mouse, capillary invasion began to occur at the periphery, and by the seventh day, vessels had grown in towards the island of hypertrophic chondrocytes. Finally, on the eighth day, when vascular in-growth had penetrated deeply into the island of hypertrophic chondrocytes, mineralization of the matrix was demonstrated by staining with alizarin. Osteoblasts deposited bone on the spicules of calcified cartilage, and in this way the ossification center began to form. These observations suggest that a factor may be synthesized by the hypertrophic chondrocyte. This factor is chemotactic for endothelial cells and being secreted into the extracellular matrix, thus inducing capillaries to grow into the cartilaginous epiphysis toward the hypertrophic chondrocytes. Besides giving a clear demonstration of the endochondral ossification process, Floyed's findings also provide us with some insights about the control of this process.

Formation of the skeleton, fracture-healing, and bone growth are three of the main fields in orthopedic study. All these processes are directly related to endochondral ossification. In patients who have disorders of bone growth such as metatrophic dwarfism, the number of columns of hypertrophic cells that are invaded by vessels is dramatically decreased. The control mechanism of osteogenesis is therefore an important field awaiting for future investigation.

2.2.3 Difficulties in Growth Plate Chondrocytes Research

During the last decade, most of the laboratory work on the cartilage research has been focused on the composition of the extracellular matrix and the molecular structure of its components (Delbruck, 1986), and very little advance has been made in the area of cartilage diseases. It seems logical to put more emphasis on the understanding of biochemical characteristics of the chondrocytes in normal cartilage, and on their responses to endogenous and exogenous factors which might disturb the dynamic equilibrium of the cartilage structures. Most of the embryonic cartilaginous skeleton is eventually replaced by bone in a process called endochondral ossification in the growth plate cartilage. Since complex biochemical processes occur in such a small anatomical area (endochondral ossification center) in a short period of time, in order to study these processes, it is necessary to prepare homogeneous samples of resting, proliferating and hypertrophic chondrocytes. Most investigators have attempted to prepare pure samples of the different zones of growth plate cartilage by microdissection. Wuthier (1969) scraped off sequential layers of calf growth plate. Kuhlman (1965) took microtome sections of dog and rabbit growth plates. The scraping technique presented problems in reproducibility and identification of layers, while microtome method required preliminary freezing. Another technique, micropuncture, allowed precise localization of the cells to be analyzed, but the potential number of analysis that could be performed was limited. Ray et al. (1982) then tried to use Ficoll density gradient to separate chondrocytes with different cell density, but this was not very successful. Most of the previous investigations used a population of mixed stages of chondrocytes (Boyan and Shaffer, 1982; Boyan, et al., 1988). It is known that cartilage cells at different stages of differentiation have different metabolic activities and respond differently to various kinds of hormones

and stimulators. An *in vitro* model using porcine growth plate chondrocytes developed in this study is able to identify and separate the cartilage cells in different stages of differentiation by applying countercurrent centrifugal elutriation (CCE) method. Using CCE, it is possible to obtain pure chondrocyte populations for more precise investigations.

2.3 METHODS

2.3.1 Isolation of Porcine Chondrocytes

Protocol concerning animal research ethics has been approved by relevant committee of our Institute. The method of O'Keefe et al. (1989) was used, with minor modifications. Chondrocytes were isolated from a pig between 4 - 5 weeks of age (about 10 kg). The animal was sacrificed by injecting 10 ml of 2.5% pentobarbital directly into its heart. The rib cage was then dissected and cleaned of connective tissue under asceptical conditions. The growth plate cartilage was taken out at the osteochondral junctions of all the ribs. The cartilage was then placed in a modified F-12 medium (magnesium-free, 0.5 mM $CaCl_2$) (Sigma, St. Louis, Missouri). After weighing, the cartilage was cut into 0.1-mm pieces and subjected to digestion with 0.1% trypsin (type III; Sigma) for 30 minutes in the modified F-12 medium at 37°C in order to break down the core and link proteins of proteglycans in the cartilage matrix. The tissue was subsequently rinsed twice with the modified F-12 medium and subjected to 0.1% hyaluronidase (type I-S; Sigma) in F-12 digestion for 1 hr to cleave the proteoglycan aggregate. This was followed by overnight digestion in 0.1% collagenase (type IIA; Sigma) in a shaking water bath at 37°C to further break down the collagen fibrils. One ml of each enzyme solution was used for every 20 mg of cartilage. After low speed centrifugation at 600 x g for 4 min., the cells in the pellets were resuspended and then filtered through a glass wool filter and washed twice with the modified F-12 medium to remove the debris of digested matrix. After cell-counting with a hemocytometer, the chondrocytes were then separated by countercurrent centrifugal elutriation.

2.3.2 Labeling of Chondrocytes for Elutriation

Chondrocytes were released from the resting zone cartilage far from the osteochondral junction of ribs. At the same time, thin growth plate cartilage layers at

the osteochondral junction were also obtained. Part of the resting zone and growth plate cartilage were retained for histological examination.

Chondrocytes from the resting cartilage were labeled with L-[2,3-^{3}H] proline (100 mCi/mmol) in the presence of 0.1 mM unlabeled proline in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 5 % fetal bovine serum (GibcoBRL, Grand Islan, New York) for 2 h at 37°C. These cells were rinsed twice with the modified F-12 medium containing cold proline (0.1 mM) to remove radioactivity that was not associated with the cells. The labeled chondrocytes were then pooled with unlabeled total growth plate cells (i.e. cells from resting zone cartilage plus cells from thin growth plate cartilage) for countercurrent centrifugal elutriation.

In some experiments, chondrocytes released from the thin layer growth plate cartilage were labeled by the same procedures as described for the chondrocytes released from the resting cartilage.

2.3.3 Countercurrent Centrifugal Elutriation (CCE)

A Beckman J-6M/E centrifuge (Beckman, Palo Alto, California), installed with JE-5.0 elutriator and Sanderson Chamber was thoroughly equilibrated with modified F-12 medium containing 5% fetal bovine serum (FBS) (GibcoBRL, Grand Island, New York). The cells were exposed briefly to DNase (Sigma, St. Louis U.S.A.) to prevent cell aggregation. About 6 x 10^7 cells were loaded onto the rotor (radius = 10.6 cm) in 20 ml of the modified F-12 medium containing 5% FBS at 4°C. Loading was performed at a constant rotor speed of 3,000 rpm. After the cells were loaded and equilibrated in the separation chamber for 10 min., elutriation was performed at various rotor speeds from 3,000 to 1600 rpm (see Table 2.1). The fluid flow was maintained at a rate of 30 ml/min throughout the procedure. Cells were

collected with two 50 ml sterilized centrifuge tubes (Corning, New York) at each rotor speed.

2.3.4 Chondrocyte Size Determination

The Coulter Counter-Channelyzer C256 system (Coulter Electronics, Hialeah, Florida) was used to determine the size distribution of the separated cells in each tube. The mean cell volumes were determined by using a calibration constant derived from latex spheres of uniform size. Standard deviations for the mean volumes and diameters of the elutriation fractions were determined by analyzing the distribution curves from the Coulter Counter.

2.3.5 Chondrocyte Cultures

Chondrocytes in the pooled elutriation fractions were plated in 24-well culture plates (Corning) at a density of 1 x 10^5 cell/cm^2 in DMEM containing 5% FBS, 50 mg/ml ascorbate (Sigma). Assays on cultured cells were performed after the cells have settled for 9 h in culture.

2.3.6 Thymidine Incorporation Assay on Cultured Chondrocytes

After the elutriated cells have settled for 9 h in monolayer culture, they were then labeled with 5 μCi/ml [^{3}H]-thymidine (40Ci/mM) (New England Nuclear) in the presence of 5 μM unlabeled thymidine as carrier in DMEM containing 5% FBS for 4 h at 37°C. The medium with excess thymidine was aspirated and the cells were washed with 1 ml 0.15 M NaCl. The cells were then lysed with 0.25 ml 0.25 M NaOH. After 20 min, attached cells were scraped out with a cell scraper. The lysed cell solution was transferred to a 5-ml plastic tube. Another 0.25 ml NaOH was used to rinse the well of the culture plate and also poured into the same tube. Alkalinity was neutralized by adding 0.5 ml of 0.25 M HCl. One ml of HEPES-Mg-Ca with 2.5

mg/ml bovine serum albumin, which acted as protein carrier, was also added to the tube. Deoxyribonucleic acid (DNA) was precipitated with 0.5 ml of 10 M perchloric acid at 4°C for 20 min. Precipitate was spun at 18,000 x g for 30 min. at 4°C . After aspirating the supernatant, 0.5 ml of 0.25 M NaOH was added to resuspend the pellet. The resuspended solution was transferred to counting vials and 4 ml of scintillation fluid was added. The radioactivity of the acid-insoluble DNA content was counted by a Beckman Liquid Scintillation Spectrometer. Standards of the radiolabeled medium were prepared for direct calculation of the incorporation of radioactive thymidine into DNA.

2.3.7 Alkaline Phosphatase Assay on Cultured Chondrocytes

The enzyme activity of alkaline phosphatase was assayed by a colorimetric method with p-nitrophenyl phosphate as the substrate (O'Keefe et al., 1989). Assays were performed directly in the culture wells containing approximately 1 x 10^5 cells/well after the isolated chondrocytes have settled for 16 h. The culture medium was aspirated from the wells, the cells were then rinsed with 0.15 M NaCl solution and the supernatant again aspirated. One ml of reaction solution containing 0.25 M 2-methyl-2-amino propanol, 1 mM $MgCl_2$, and 2.5 mg/ml p-nitrophenyl phosphate (Sigma) at pH 10.3 was then added to the wells at 37°C to initiate the reaction. The reaction was stopped by adding 0.5 ml 0.3 M trisodium phosphate, pH 12.3, the absorbance at 410 nm was measured using 0.15 M NaOH as blank. The activity of alkaline phosphatase was calculated from a standard curve prepared by using p-nitrophenol and expressed as mM/10^6 cells/h.

2.3.8 Sulfate Incorporation Assay on Cultured Chondrocytes

In this assay, cell cultures were exposed to 12 μCi/ml of $^{35}SO_4^{2-}$sodium salt (DuPont NEN, Boston) in DMEM, which contained 0.814 mM carrier sulfate, for 4 h at 37°C.

After incubation, the medium was transferred into a small plastic tube. Cells were lysed by adding 0.25 ml of 0.25 M NaOH to each well and left for 20 min. Cells were then scraped out with a cell scraper and were transferred to the same plastic tube. Another 0.25 ml of 0.25 M NaOH was used to rinse the wells of the culture plate and transferred to the tube. After adding 0.75 ml of 0.15M NaCl, the solution was dialyzed against phosphate buffered saline, pH 7.4, using 12,000 - 14,000 MW dialysis tubing (Spectrum Medical , Los Angeles) in order to remove unincorporated radiolabeled sulfate. Phosphate buffered saline were changed twice a day. After 3 days, 0.75 ml of the sample inside the dialysis tubing was placed into a scintillation vial and into which 4 ml of scintillation fluid was added. The radioactivity of $^{35}SO_4^{2-}$ incorporated macromolecules was quantified by a Beckman scintillation spectrometer. Data are expressed as nmol of sulfate incorparation per 10^6 cells per hour (nmol/10^6 cells/h)

2.4 RESULTS

Porcine rib is an ideal model to study endochondral ossification since its growth plate chondrocytes differentiate in a very regular manner as demonstrated in Figure 2.1 which shows the histological section of porcine rib growth plate. The cartilage far from the osteochondral junction is the resting zone cartilage containing homogeneous small resting chondrocytes. At about 1 mm from the junction, unidirectional chondrocyte divisions occur and stacks of proliferative chondrocytes can be seen. At the front of these stacks facing the junction, chondrocytes become hypertrophic and the largest chondrocytes are located adjacent to the bone (Figures 2.1 a &b). Figure 2.2 shows the histological sections of a growth plate slice for chondrocytes isolation. A smooth mineralized and vascularized zone separates the bone and cartilage tissue. When force was applied to both ends of the rib, the cartilage tissue containing the entire growth plate detached easily from the mineralized bone tissue. A thin slice of growth plate cartilage, approximately 1.5 mm thick, containing all the hypertrophic and proliferative chondrocytes, part of the resting chondrocytes, and some red blood cells attached on the fracture site (Figure 2.2a), could then be dissected. At the same time, pure resting chondrocytes were obtained by dissecting tissue from the resting zone cartilage (Figure 2.2b).

After sequential enzymatic digestion, chondrocytes released from the growth zone cartilage were heterogeneous while chondrocytes released from the resting zone cartilage were homogenous in size and appearance (Figure 2.3 a & b). Typically, the yield of resting and growth plate chondrocytes were about 5 and 6 x 10^7 cells per gram of cartilage respectively. The viability of both types of released chondrocytes were not less than 85 %.

When isolated growth plate chondrocytes were loaded into the Beckman J-6 M/E countercurrent centrifugal elutriation (CCE) system (Table 2.1), 18 elutriated cell fractions were collected with sterilized tubes. The cells obtained in each fraction were spherical and quite homogeneous in size (Figure 2.4 a & b). The recovery rate of the elutriation procedures was 80 %. The mean cellular volume and the total cell number of each fraction were analyzed. The first elutriated fraction contained the smallest cells and the cell sizes increased steadily in the proceeding fractions. The mean cell volume of the smallest chondrocyte fraction is 767 femtoliter (fl), and that of the largest is 2859 fl. The cell distribution of chondrocytes of the elutriated fractions is also listed on Table 2.1. The cell numbers rose dramatically in the first 7 fractions and then decreased sharply at the next two fractions. From the tenth fraction, it declined steadily until the end fraction. From a plot of the cell numbers versus the mean cell volumes, a clear picture of the cell size distribution for the elutriated chondrocytes emerged (Figure 2.5). Cells with volumes ranging from 800 fl to 1200 fl represented nearly half of the total elutriated growth plate chondrocyte population.

In order to identify the resting and hypertrophic chondrocyte subpopulations from the total elutriated growth plate chondrocyte fractions, the cell sizes of chondrocytes released from the resting zone cartilage and chondrocytes released from the thin growth plate cartilage were determined. The minimum mean cell volume of released resting zone cartilage chondrocytes was not less than 212 fl and the maximum not larger than 1518 fl (Figure 2.6a). For the chondrocytes released from growth plate cartilage, two populations were detected (Figure 2.6b). The mean and maximum cell volume of the first population of cells were 32 fl and 171 fl respectively, which were confirmed to be red blood cells by light microscopy. The second population had cell sizes ranging from 1583 to 3652 fl and represents the hypertrophic subpopulation.

To further confirm that elutriated chondrocyte fractions with cell volume smaller than 1500 fl are the resting chondrocyte subpopulation, cells released from resting zone cartilage were labeled with tritiated proline. After pooling this radioactive labeled resting chondrocytes with the total growth plate chondrocytes, the cells were subjected to CCE. Radioactivities of the various elutriated fractions were counted by a scintillation spectrometer (Figure 2.7). Radioactivities of the first three fractions increased sharply and those of the proceeding fractions declined rapidly. Only minimum amount of radioactivity could be detected for those fractions with cell volumes larger than 1500 fl.

To confirm that the fractions containing elutriated cells of larger size were really composed of hypertrophic chondrocytes, cells released from thin layers of growth plate cartilage adjacent to the osteochondral junction were labeled with tritiated proline. After pooling these cells with unlabeled chondrocytes released from resting zone cartilage sections, the cell mixture was separated by elutriation. Radioactivities of the various elutriated fractions were recorded. As shown in Figure 2.8, elutriated chondrocyte fractions with cells smaller than 1600 fl showed very low radioactivities. The radioactivities increased sharply as the cell volume increased until a peak was reached at the 2700 fl fraction. A slight decrease then followed.

Chondrocytes in the pooled elutriation fractions were plated in 24-well culture plates and settled for 9 h before conducting the assays. Prolonged culture of the elutriated chondrocytes have also been performed. It was found that all the chondrocytes could maintain spherical shapes as those of freshly isolated cells for the first 24 h. The cells then became flattened. When the medium were replaced with fresh medium every two days, the cultures became confluent after 7 days. The confluent resting

chondrocytes were granular, thick and polygonal in shape while the hypertrophic cell were thin and irregular.

Figure 2.9 shows the alkaline phosphatase activities of cultured chondrocytes in various elutriated fractions. Alkaline phosphatase activities were maintained at a minimum detectable level in the fractions of cells smaller than 1600 fl. The enzyme activities increased as the cell size increased. A plateau of activity was observed in fractions of cells with volume larger than 2000 fl.

The thymidine incorporation rates of cultured chondrocytes in various elutriated fractions was shown in Figure 2.10. The rate of thymidine uptake was very low in the chondrocyte subpopulations with volume smaller than 1518 fl. It increased drastically from about 75 - 275 fmol/10^6cell/h for the fractions larger than 1518 fl with a peak at the 2100 fl fraction. Then the rate declined to 25 fmol/10^6cell/h again.

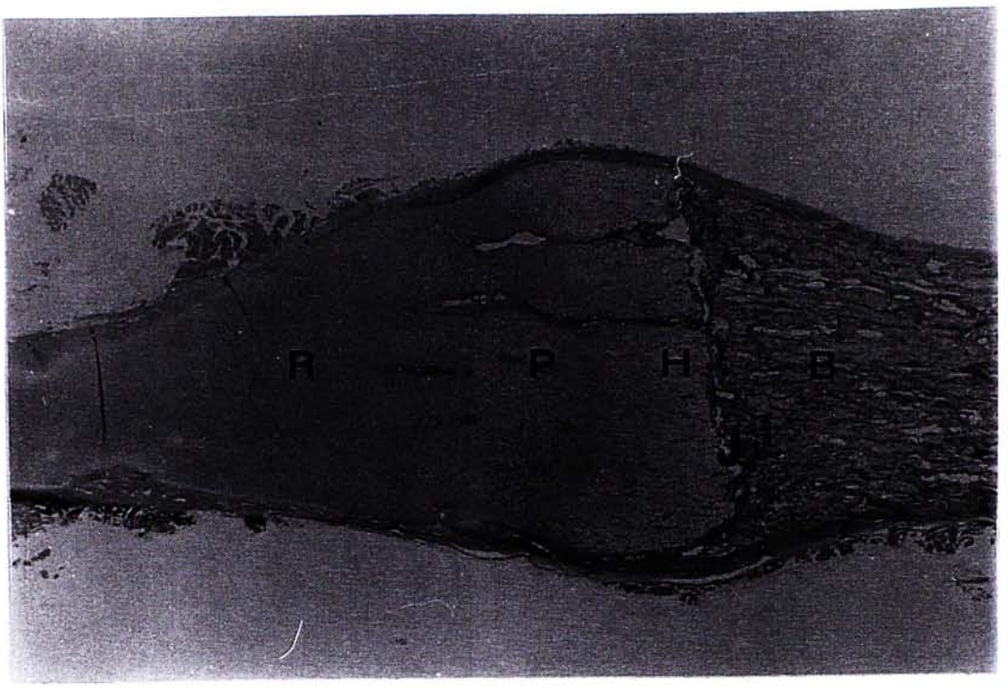
Figure 2.11 shows the sulfate incorporation of cultured chondrocytes in various elutriated fractions. For cells with volume less than 1400 fl, sulfate incorporation rate was around 1 nmol/10^6 cells/h. The rate then rose as the cell size increased and reached a peak of 5 nmol/10^6cells/h for cells at 2300 fl. A slight decrease then followed.

Table 2.1 Porcine Chondrocyte Elutriation Profile

Elutriation Fraction	Rotor Speeds (rpm)	No. of Cells (millions)	Mean Cell Volume (fl)
1	2900	0.61	767
2	2800	0.44	810
3	2700	0.87	832
4	2600	2.17	853
5	2500	4.01	886
6	2400	6.35	927
7	2300	7.17	981
8	2250	5.60	1042
9	2200	4.00	1196
10	2150	3.04	1388
11	2100	2.75	1569
12	2050	2.76	1749
13	2000	2.09	1878
14	1950	1.68	2073
15	1900	1.49	2326
16	1850	0.95	2668
17	1800	0.56	2796
18	1700	0.63	2859

About 6×10^7 porcine chondrocytes were loaded onto the rotor with radius = 10.6 cm of a Beckman J-6 M/E centrifuge. Elutriation was performed at various rotor speeds (in rpm) as indicated.

a.



b.

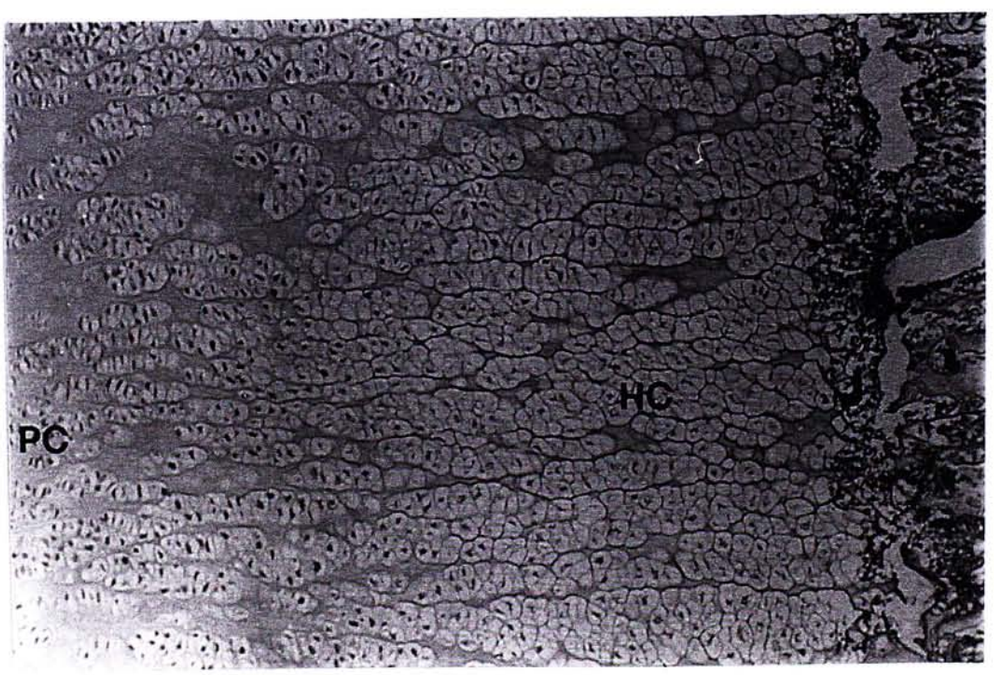
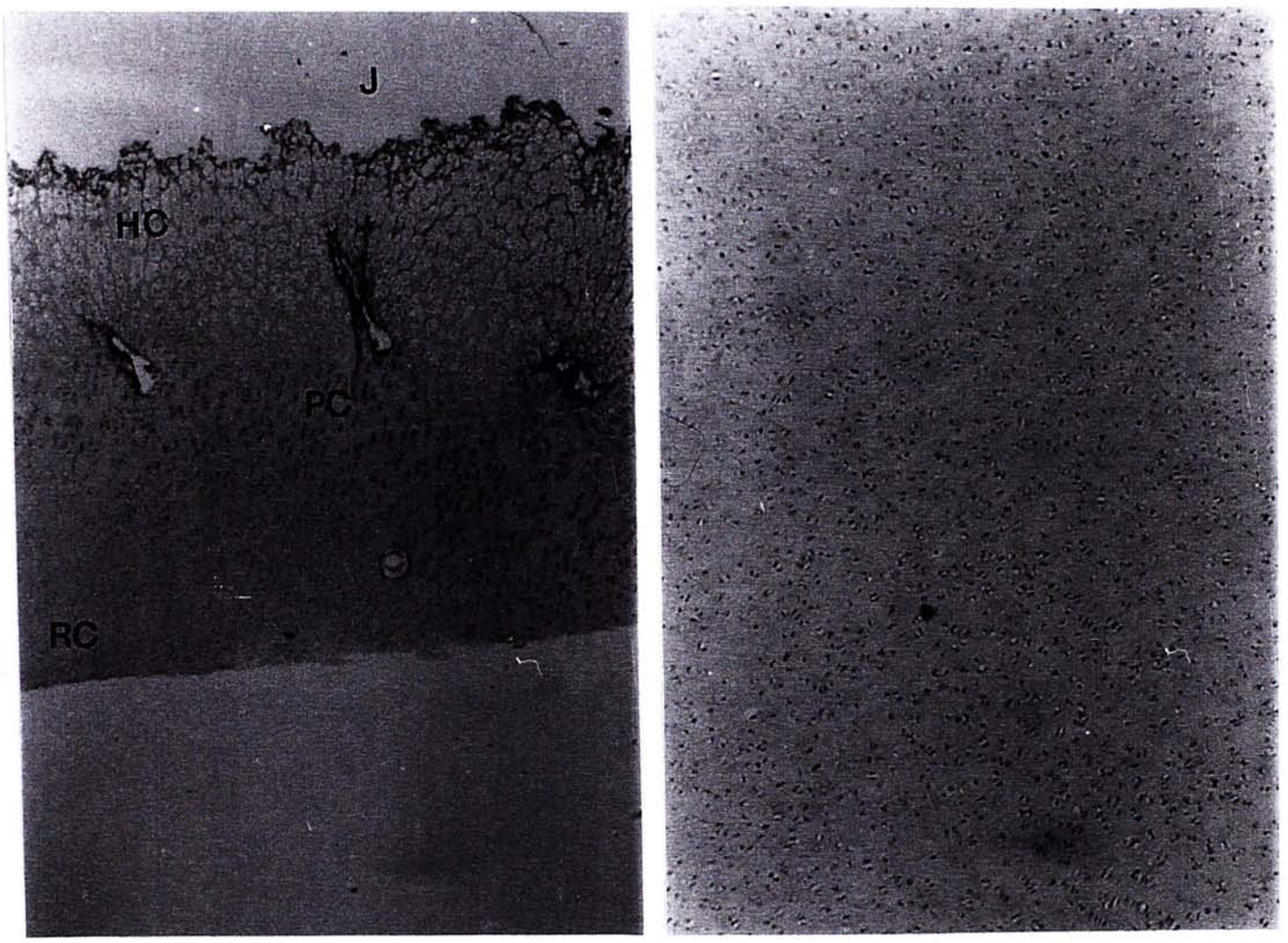


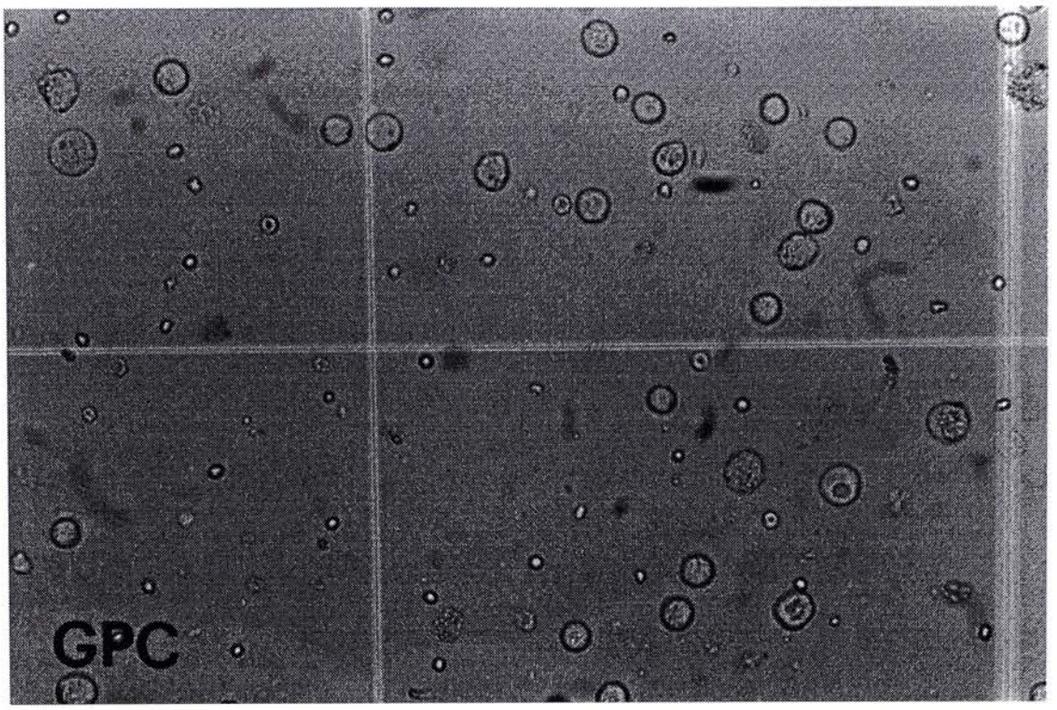
Figure 2.1 (a) Low power micrograph showing the pig rib growth plate. Bone (B) and cartilage are connected at the osteochondral junction (J). The hypertrophic zone (H), proliferative zone (P) and resting zone (R) are arranged in a very regular manner (H&E, original magnification x 10). (b) Micrograph at high power magnification showing the osteochondral (J), the hypertrophic chondrocytes (HC) and proliferative chondrocytes (PC) can be clearly observed (H&E, 63x)



a. b.

Figure 2.2 Histological sections of growth plate slice for chondrocyte isolation. a. A growth plate slice, dissected from pig rib for elutriation, showing presence of hypertrophic chondrocyte (HC), proliferative chondrocytes (PC) and part of resting chondrocytes (RC). Some red blood cells were observed at the edge of the osteochondral junction (J) (H&E, 25x). b. Resting zone cartilage for resting chondrocyte release (H&E, 25x).

a.



b

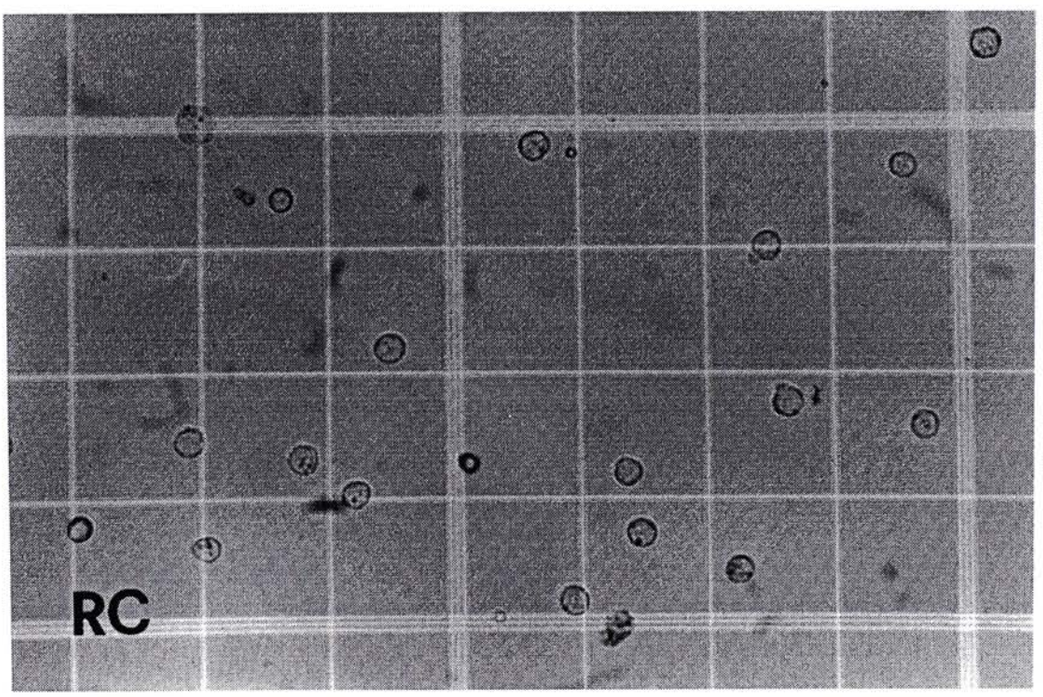
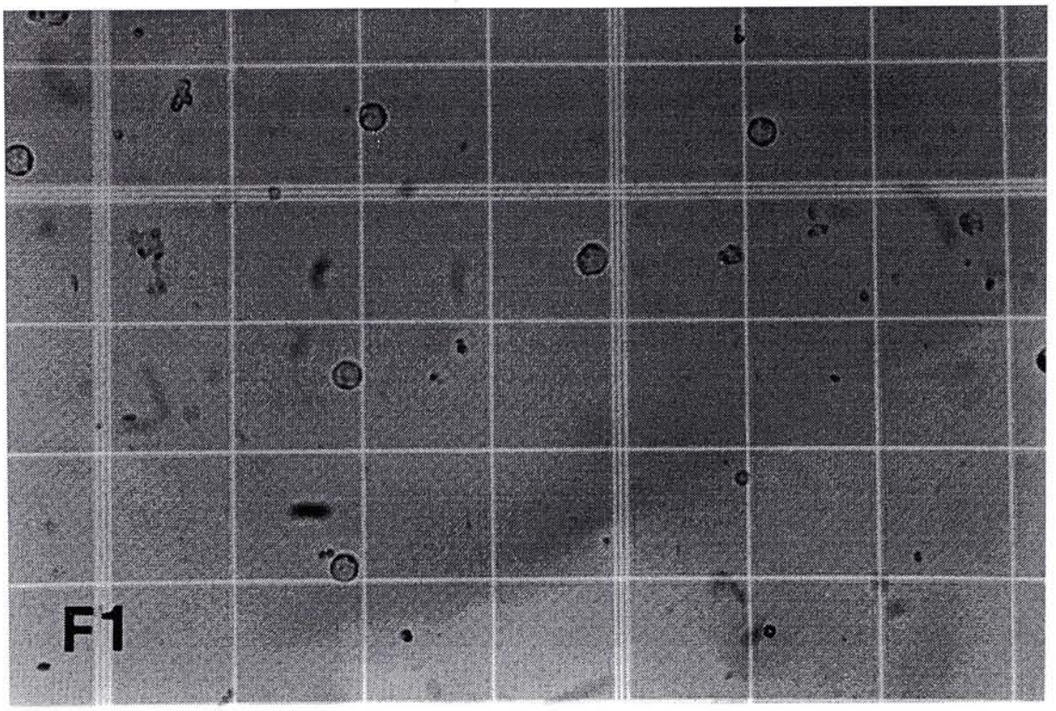


Figure 2.3 Chondrocytes released from growth plate zone cartilage were heterogeneous in size (a) while those from resting cartilage were small and homogeneous (b). (250x)

a



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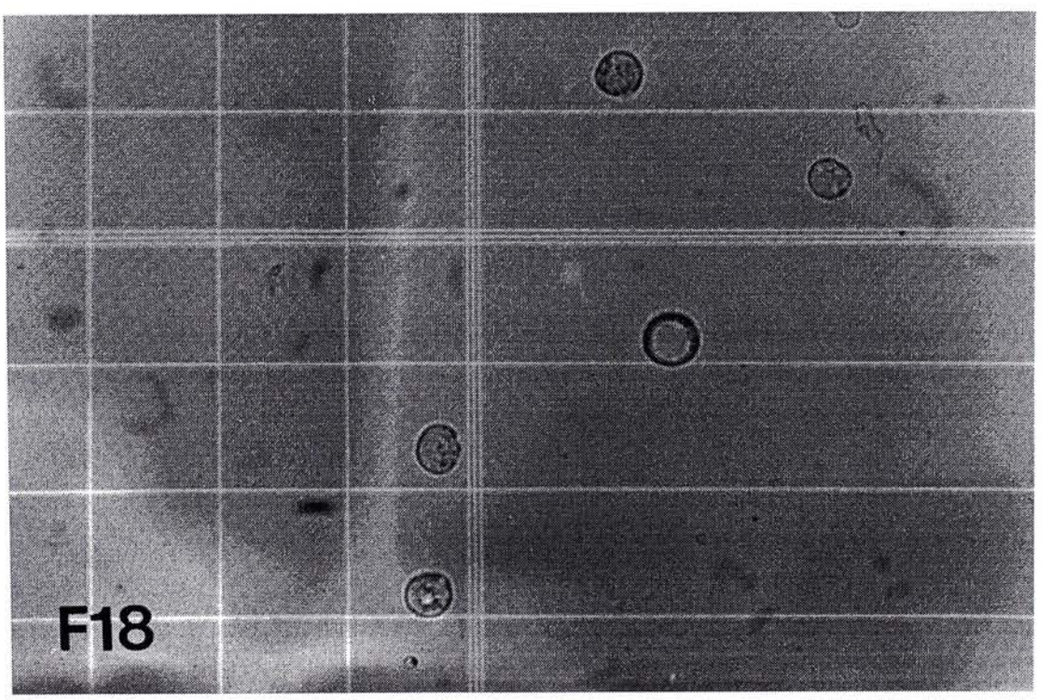

Figure 2.4 Chondrocytes collected from the first (a) and eighteenth (b) elutriated fractions. The mean cell volume of the first chondrocyte fraction was 600 fl, and that of the eighteenth was 2700 fl, which was 4.5 times larger. (250x)

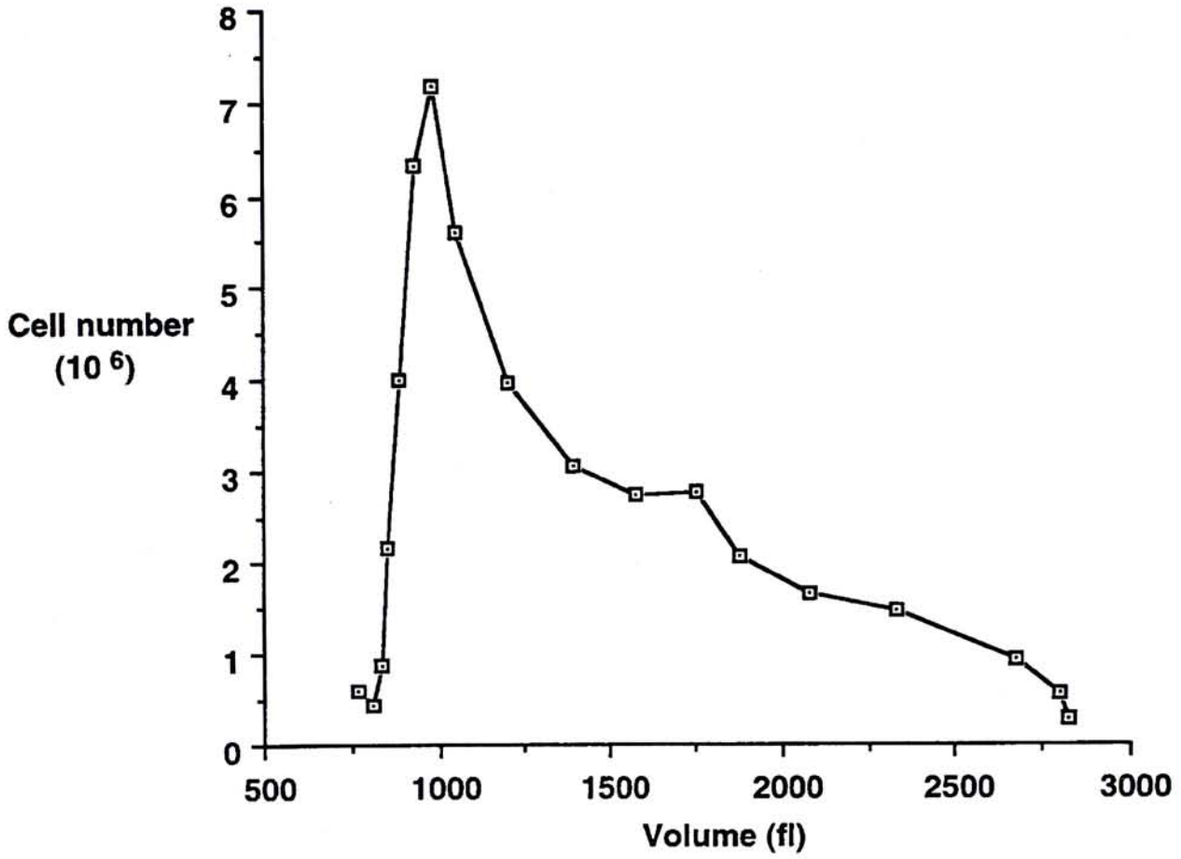

Figure 2.5 Size distribution of the elutriated growth plate chondrocytes. Cellular volumes were determined by Coulter Counter- Channelyzer C256 System as described in Methods section 2.3.

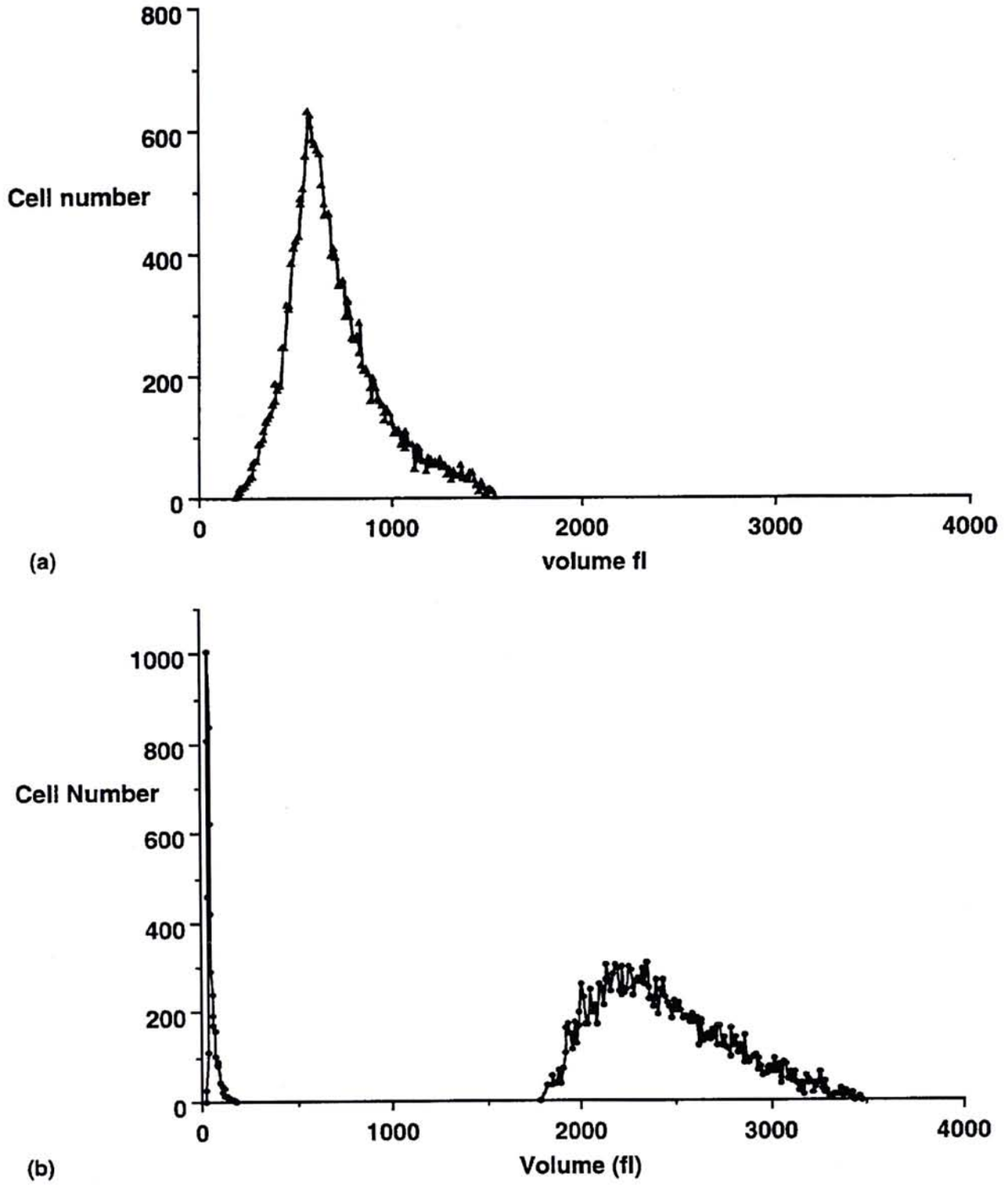

Figure 2.6 Cell size distribution of freshly isolated chondrocytes. (a) Chondrocytes from resting zone cartilage: the minimum, mean and maximum cellular volume of chondrocytes released were 212, 520 and 1518 fl respectively. (b) Chondrocytes released from thin layer of growth plate cartilage adjacent to the osteochondral junction: the first population had maximum, mean and miniumum cell volume of 15, 32 and 171 fl respectively; the second population had minimum, mean and maximum cell volume of 1583, 2431 and 3652 fl respectively.

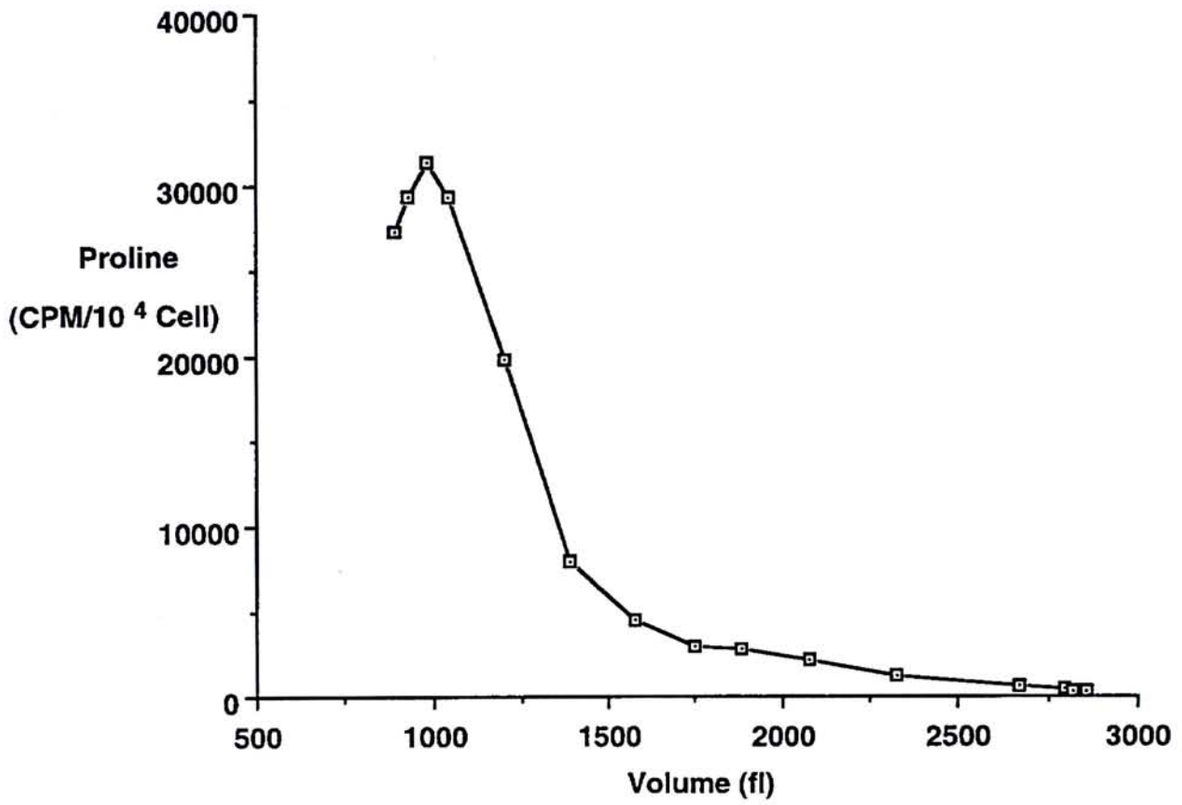

Figure 2.7 Elutriation of labeled resting chondrocytes. Isolated chondrocytes from resting zone cartilage were labeled (as described in Methods section 2.3) and then pooled with unlabeled chondrocytes from growth plate zone cartilage. The radioactivities of each elutriated fraction were recorded. Each data-point represents the mean of four measurements. The C.V. was less than 1%

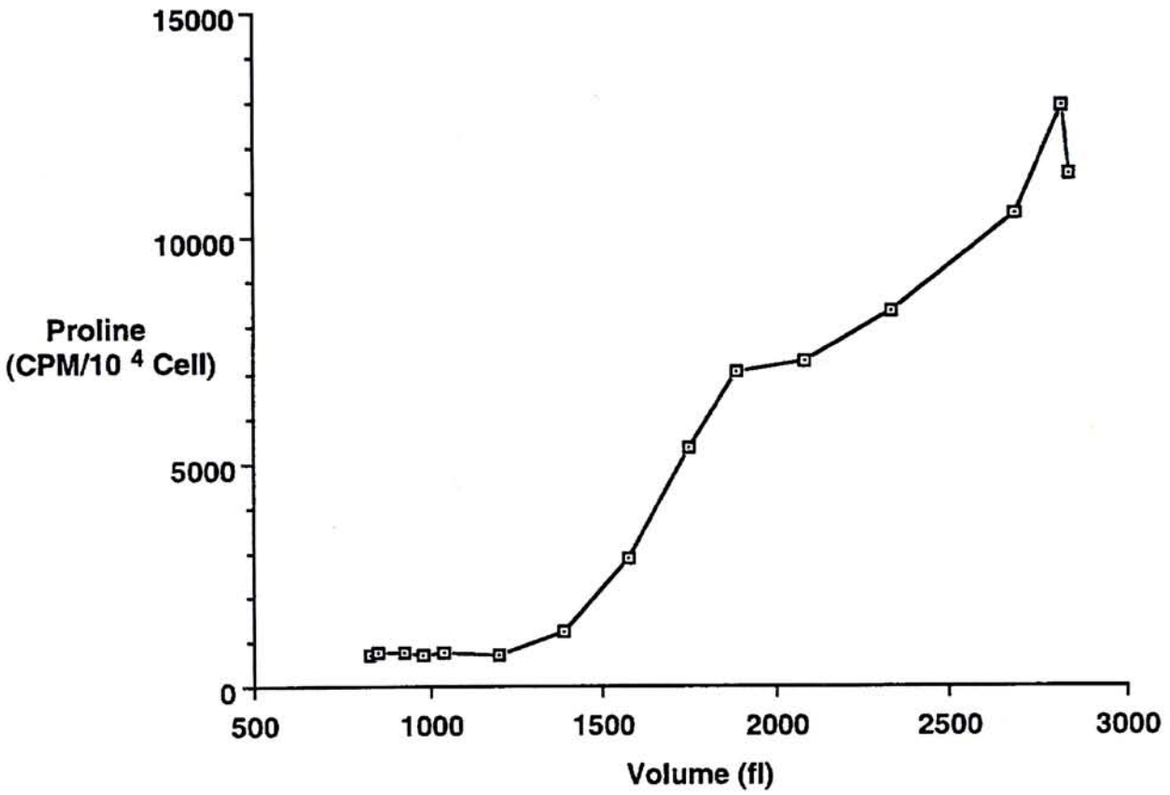

Figure 2.8 Elutriation of labeled hypertrophic chondrocytes. Isolated chondrocytes from thin layer growth plate cartilage adjacent to the osteochondral junction were labeled (as described in Methods) and then pooled with unlabeled chondrocytes from thich growth plate cartilage. The radioactivities of each elutriated fraction were recorded. Each data-point represents the mean of four measurements. The C.V. was less than 1%.

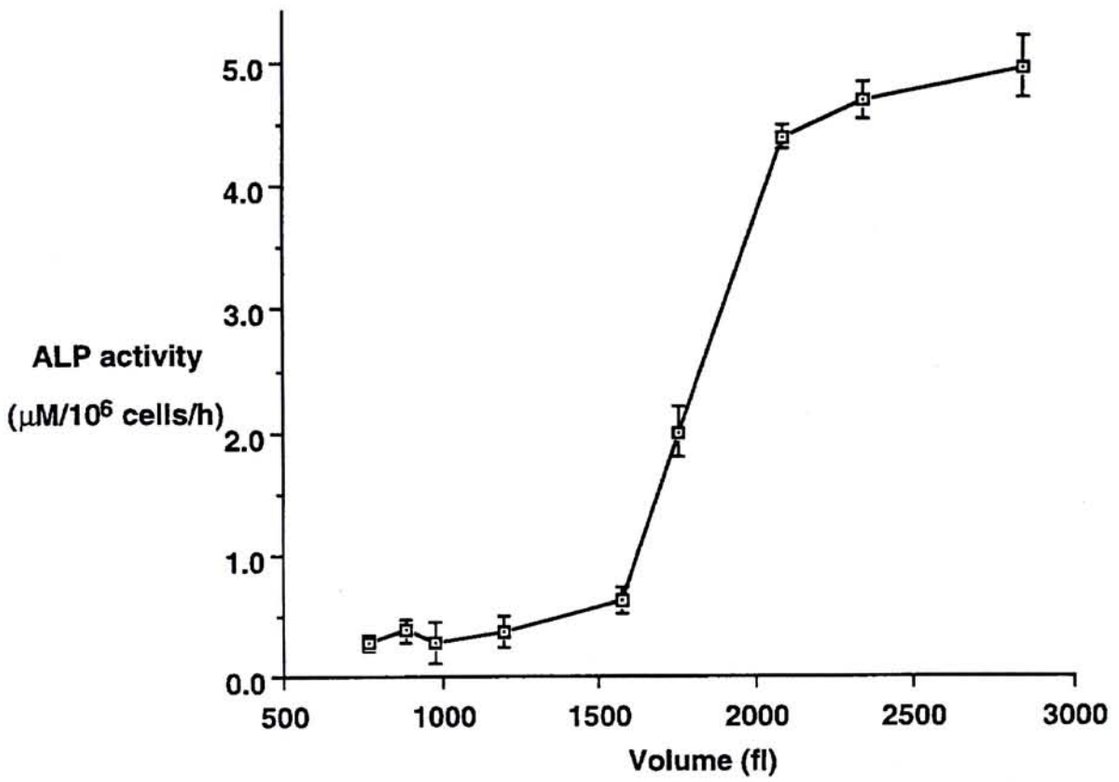

Figure 2.9 Alkaline Phosphatase (ALP) activities of cultured chondrocytes in various elutriated fractions. The enzyme activities were measured after 9 hr of cultivation (as described in Methods). Data are expressed as mean ± S.D. (n=4).

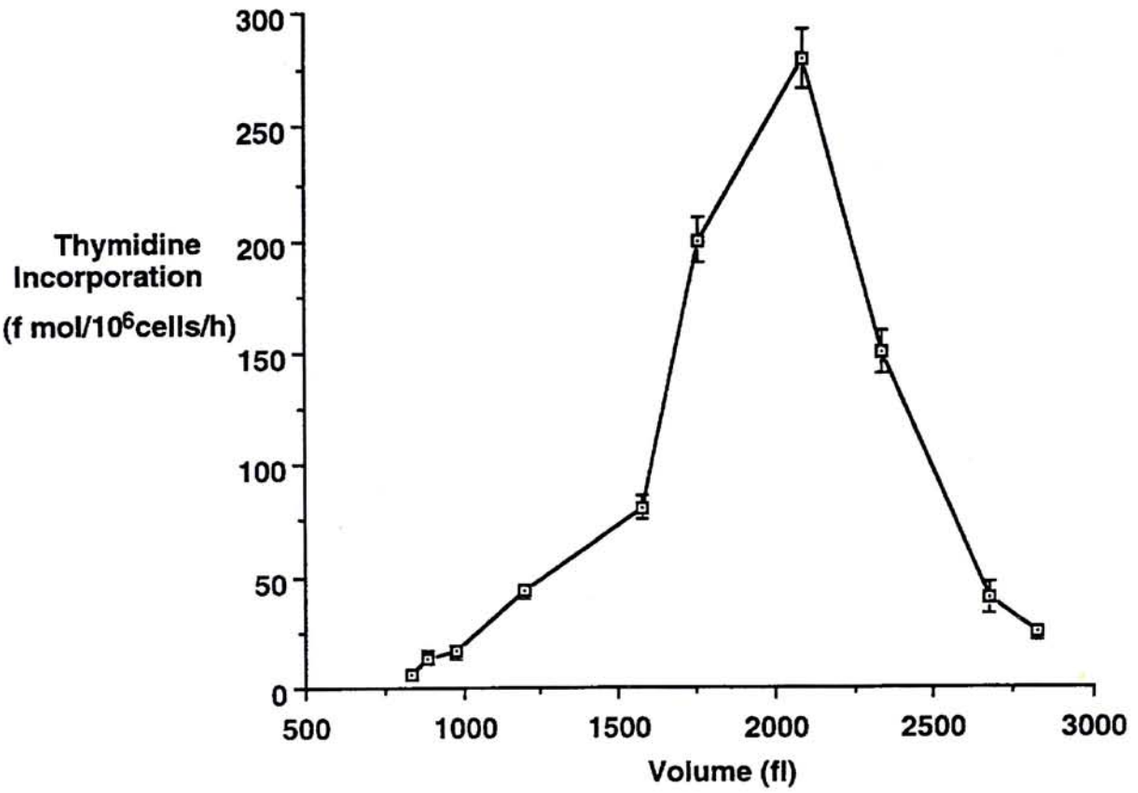

Figure 2.10 Incorporation rates of thymidine of the cultured chondrocytes in various elutriated fractions. The incorporation rates of radiolabeled thymidine were determined after 9 hr of cultivation (as described in Methods). Data are expressed as mean ± S.D. (n=4).

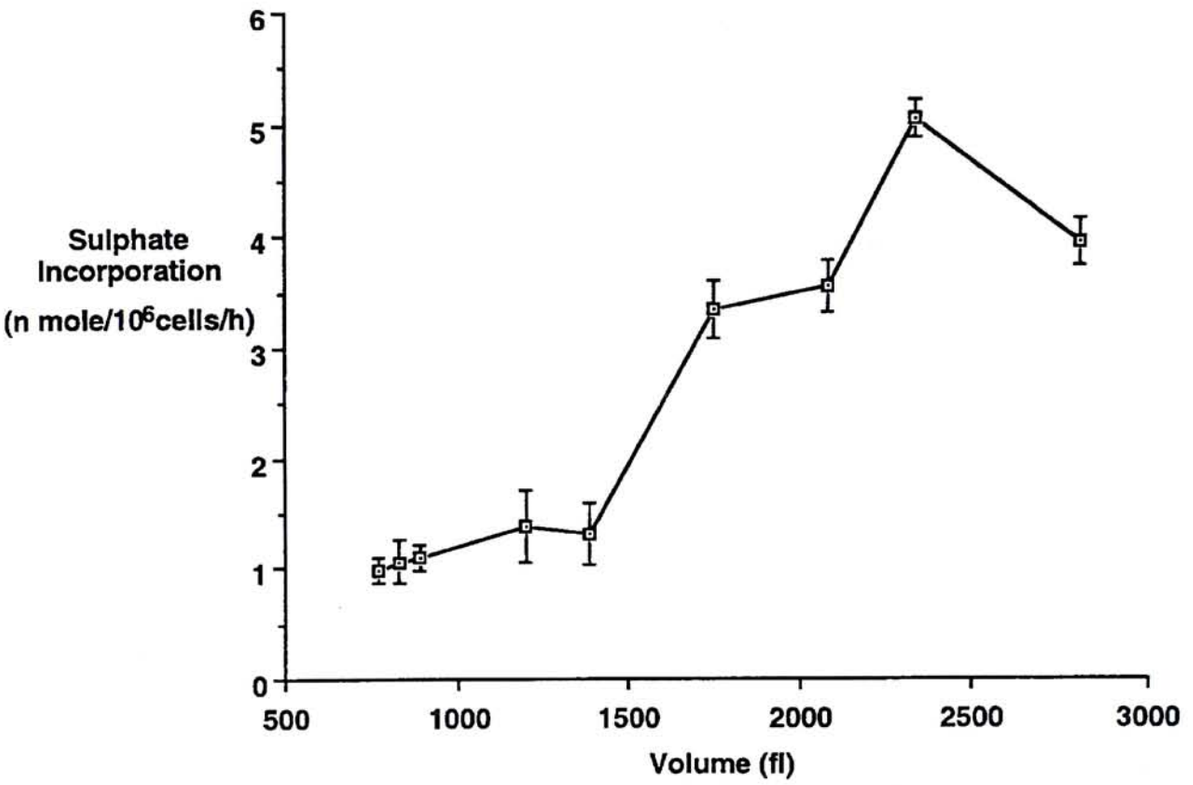

Figure 2.11 Sulphate incorporation rates of the cultured chondrocytes in various elutriated fractions. The incorporation rates of radiolabeled sulphate were determined after 9 hr of cultivation (as described in Methods). Data are expressed as mean ± S.D. (n=4).

2.5 DISCUSSION

Growth-plate chondrocytes from chick have been extensively studied and characterized. These cells are basically classified into three subpopulations based on their histological and functional differences (O'Keefe et al., 1989). The cartilage cells located farthest away from the osteochondral junction are the resting chondrocytes. These cells are small in size and homogeneous in appearance and represent the quiescent form of the growth-plate chondrocytes. Next to the resting chondrocytes are proliferative chondrocytes. These are the most active cells which are constantly involved in cell proliferation. The cells adjacent to the osteochondral junction are the hypertrophic chondrocytes. These hypertrophic cells increase in their size as they approach the osteochondral junction. The distribution pattern of chondrocytes in the growth plate cartilage were similar in both chicken (O'Keefe et al., 1989) and pig as shown in the present study (Figure 2.1a & b, figure 2.2a & b).

Due to technical limitations in separating growth plate chondrocytes into subpopulations at different differentiating stages, many characteristics described in earlier studies by many researchers could be the total or combined effect of a mixture of chondrocytes in different maturation stages rather than their true individual features. At the same time, red blood cell contamination often complicates the data interpretation. Much of the recent findings suggested that cells in different differentiating stages have distinct physiological, biochemical and morphological features (Rosier et al., 1989; O'Keefe et al., 1990; Gunter et al., 1990). As a result, separation and identification of different subpopulations become critical issues for studying growth plate chondrocytes as well as endochondral ossification.

In the present study, we have characterized the porcine growth plate chondrocytes. With a yield of approximately 6×10^7 cells per gram of cartilage from each animal, the porcine rib growth plate provides an ideal source of chondrocytes for studying endochondral ossification. Moreover, pure sample of resting chondrocytes can be obtained with relative ease from the resting zone cartilage since they are situated away from the growth plate (Figure 2.2).

Countercurrent centrifugal elutriation (CCE) is a powerful cell separation technique which has been successfully applied by workers in cancer research for many years (Grading and Meistrich, 1980; Lord & Keng, 1984). In 1989, this technique has been applied for the first time on avian growth plate cells by Rosier and his co-workers (O'Keefe et al., 1989). In the present study, this method was modified to apply on a mammal model. Results obtained from our investigation showed that porcine growth plate chondrocytes can be effectively separated by size differences using CCE (Table 2.1 and Figure 2.4).

The cell size distribution of freshly isolated chondrocytes was determined. The results showed that the maximum cell volume of the resting chondrocyte population is smaller than 1518 fl (Figure 2.6a) and the cell sizes of hypertrophic chondrocytes ranged from 1583 fl to 3652 fl (Figure 2.6b). To confirm these findings, cells released from resting zone cartilage were labeled with tritiated proline and pooled with unlabeled chondrocytes released from the respective cartilage sections. When the cell mixture was then separated by elutriation, results showed that radioactivities of those fractions with cells larger than 1500 fl were very low (Figure 2.7). Similarly, when mixture of labeled and unlabeled cells of thin layers of growth plate cartilage were subjected to CCE, only cells with size larger than 1600 fl exhibited radioactivities (Figure 2.8).

The alkaline phosphatase (ALP) activities of the individual fractions of chondrocytes after elutriation were determined. ALP is an important maturation marker of chondrocytes and its activity increases in the hypertrophic zone of the growth plate (Kuhlman, 1965; Lewinson et al., 1982). Although its role is not fully understood, ALP is essential for calcification of the matrix (Fallon et al., 1986). Extensive investigations carried out by other workers suggest that this enzyme plays an important role in both bone formation (Farley and Baylink, 1986; Wlodarski and Reddi, 1986) and extracellular matrix mineralization (Fauran-Clavel and Oustrin, 1986; Register et al., 1986). However, the mechanism by which the enzyme promotes calcification in mineralizing tissues, i.e., bone and cartilage, is poorly understood. De Bernard et al. (1986) provided evidence that cartilage-derived ALP binds calcium ions with high affinity. Results from this study (Figure 2.9) showed that elutriated porcine chondrocytes exhibited ALP activities differentially. For example, hypertrophic chondrocytes have the highest level of activity. The increase of enzyme activity was about 7 folds in hypertrophic chondrocytes compared with resting chondrocytes. This difference could not be accounted for by the greater surface area of the plasma membrane in the hypertrophic chondrocytes. This is because the increase in volume of 4.5 folds corresponds only to an increase in cellular surface area of 2.2 folds. The difference in enzyme activities is therefore not due solely to the increase in total area of plasma membrane in the hypertrophic chondrocytes, but will be due to a specific biochemical change associated with cellular hypertrophy. The content of this enzyme per unit area of membrane may increase as chondrocytes become more mature in the growth plate. The present finding is consistent with earlier observations in bovine (Grant et al., 1985) and avian chondrocytes (O'Keefe et al., 1990).

Thymidine incorporation rate is the primary indicator of DNA synthesizing activity as well as cell proliferative potential (Puzas and Brand, 1986; Puzas and Felter,

1988). Figure 2.10 shows that thymidine incorporation rates varied with changes of the mean cellular volumes of elutriated chondrocytes. The smaller cells showed low incorporation rate, indicating the minimum proliferative potential of the resting cells. The thymidine incorporation rate rose sharply starting from the fraction with cell size of 1518 fl and subsequently declined quickly from the 2100 fl fraction. From this result, we can conclude that those fractions of elutriated chondrocytes with mean cell volume just larger than the resting chondrocytes, are proliferative chondrocytes which are active in DNA synthesis. Our findings are consistent with those of Rosier et al. (1989) who show that avian proliferative chondrocytes are also active in DNA synthesis.

In order to characterize the proteoglycan synthesis ability of the chondrocyte subpopulations isolated from CCE, sulfate incorporation assay was performed. Sulfate containing glycosaminoglycans, such as chondroitin sulfate and keratan sulfate, are the predominant carbohydrate moieties in cartilage matrix proteoglycan. Thus, the incorporation rate of radioactive sulfate into cellular and extracellular macromolecules is a good indication of proteoglycan synthesis (O'Keefe et al., 1989). Data obtained here show that the synthetic rate of proteoglycan was highest in the larger chondrocytes (Figure 2.11). In addition, results of these experiments indicate clearly that chondrocytes with mean volume less than 1400 fl exhibited a low proteoglycan synthesizing rate whereas those cells with increasing cell sizes exhibited a higher rate. It is likely that cellular proteoglycan synthesis may be triggered by the onset of chondrocyte differentiation. Although the terminally differentiated hypertrophic chondrocytes showed a decline in proteoglycan synthesizing rate (Figure 2.9), they are still metabolically active even after they were isolated and plated in culture plates as primary monolayer cultures when compared to the resting cell subpopulation.

During our study, it has been observed that cultured porcine chondrocytes maintained their initial phenotypic characteristics found in freshly separated cells. Previous investigations on chick cartilage also demonstrate the ability of cultured chondrocytes in maintaining their specific characteristics (Prins et al., 1982). Monolayer cultures, established after 16 hours of cultivation *in vitro*, demonstrate the chondrocyte phenotype both morphologically and biochemically, even after growth to confluence (O'Keefe et al., 1989, 1990). Our findings in present study also with the previous findings which showed that the chondrocytes from different regions of the growth plate maintain a specific cellular subphenotype (Skantze et al., 1985; Wier and Scott., 1986). Such findings bear important implications as they permit the *in vitro* study of subpopulations of chondrocytes in culture. By using a particular phenotypic subpopulation of chondrocytes, specific interventions or stimuli can be evaluated with special reference to their ability in promoting or inhibiting maturation. Thus, culturing of chondrocytes *in vitro* which have been separated by CCE may be an useful tool for the investigation of hormones or growth factors which control endochondral ossification. Actually, we have found that porcine chondrocytes elutriated in our study could be maintained in culture medium as confluent cultures. Histomorphometric studies have demonstrated that individual hypertrophic cells are responsible for the production and maintenance of the increased areas of matrix (Hunziker et al., 1985). This finding suggests an increase in metabolic activity by these cells and is supported by the observation of increased numbers of mitochondria and secretory organelles on a subcellular basis (Buckwalter et al., 1986). In the present study, the rates of synthesis of DNA and proteoglycan in chondrocytes were measured (Figures 10 and 11). Proliferative and hypertrophic chondrocytes were found to be metabolically active cells and can synthesize large amount of proteoglycans. Therefore, the increase in size of the hypertrophic chondrocytes is not due to swelling which represents the preceding of cell death, but is due to the result of an active process.

In conclusion, by using porcine rib growth plate chondrocytes and applying the technique of CCE, effective isolation and identification of various subpopulations of the growth plate chondrocytes were achieved. CCE was found to be a reproducible and accurate cell separation technique which effectively eliminate the problem of red blood cell contamination during cell isolation in our study. Three distinct types of chondrocytes were identified and characterized in this study, each with their own morphological and biochemical characteristics. CCE was firstly used to separate growth plate chondrocytes in chick (O'Keefe et al., 1989; Rosier et al., 1989; O'Keefe et al., 1990). The present study represents an attempt in using this technique to separate mammalian growth plate chondrocytes. It is of interest to investigate whether this technique can also be used in other mammalian systems, especially in human. We believe that data on study of biochemistry of the chondrocytes can provide valuable information on the process of ossification and further studies should give an insight into the etiology of various bone diseases, such as cartilaginous tumors, fracture callus formation and heterotopic ossification.

Chapter Three

Differential Expression of Glycoconjugates during Endochondral Ossification in Porcine Growth Plate

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3.1 AIMS OF STUDY

Cartilage is the major structural component of the embryonic skeleton. Most of the embryonic cartilaginous skeleton is eventually replaced by bone and marrow in such a process called endochondral ossification, but some cartilage persists into adult life. During endochondral ossification, chondrocytes undergo a programmed sequence of biochemical and morphological changes. This process occurring in the endochondral ossification center (fetus) or growth plate (young animal) is the primary mechanism responsible for longitudinal bone growth in mammals including human.

The growth plate can be morphologically divided into 5 zones: (1) The **resting zone** consists of hyaline cartilage without morphological changes in the homogeneous chondrocytes. (2) The **proliferative zone** where the chondrocytes divide rapidly and form parallel rows of stacked cells along the long axis of the bone. (3) The **hypertrophic cartilage zone** where the chondrocytes become hypertrophic and accumulate glycogen in their cytoplasm. The resorbed matrix is reduced to thin septa separating the rows of chondrocytes. (4) In the **calcified cartilage zone**, the thin septa of cartilage matrix between rows of chondrocytes become calcified by the deposition of hydroxyapatite. (5) The **ossification zone** where calcification and vasualization occur, and the cartilage matrix is replace by bone tissue. Blood capillaries together with the undifferentiated cells originating from the periosteum invade the cavities in the cartilage left behind by the dead chondrocytes. The undifferentiated cells develop into osteoblasts, which in turn form a discontinuous cell layer over the septa of calcified cartilage matrix. Over these septa, the osteoblasts lay down the bone matrix.

Glycosylation of proteins is one of the most important post-translational modifications preceding or concomitant with their incorporation into various cytoplasmic compartments as well as organelles and their functional activation or secretion (Barondes, 1984; Hakomori, 1985). In contrast to other forms of post-translational modifications such as sulfation or phosphorylation, glycosylation is

usually quite complex and may involve sequential action of multiple enzymes leading to the formation of highly heterogeneous glycoproteins. Glycosylation determines many of the final structural and functional properties of proteins, as well as confers to them their essential biological attributes, and directs their turnover and transcellular traffic (Hakomori, 1985; Yogeeswaran, 1983). The regulation of glycosylation is important for interaction between cells and their environment during development and growth (Stanley, 1987).

The main objective of the present study is to localize and partially characterize the glycoconjugates expressed by the different subpopulations of chondrocytes in the porcine growth plate by lectin histochemistry. Lectins are specific sugar binding proteins of non-immune origin. A panel of lectins with different sugar binding specificities were used. The information on the differential expression of glycoconjugates in the growth plate chondrocytes obtained in this study can provide us a number of avenues on the separation and identification of chondrocyte subpopulations in various maturation stages. This can be achieved according to their different expression of membrane glycoconjugates using various methods such as lectin affinity chromatography and flow cytometry with fluorescein-labeled lectin probes. The separated chondrocyte subpopulations will be further characterized as part of our future project. The findings will provide us a better understanding of the process of endochondral ossification and normal bone development.

3.2 LITERATURE REVIEW

According to the definition proposed by Goldstein and accepted by the Nomenclature Committee of the International Union of Biochemistry (Goldstein et al., 1980), lectins are carbohydrate-binding proteins of non-immune origin which agglutinate cells and / or precipitate glycoconjugates. These proteins bear at least two sugar binding sites, have no enzymatic activity, may be soluble or membrane bound, and can be bacterial, animal, or plant in origin. The term agglutinin is the most common synonym for lectins since hemagglutination of blood cells still represents the most widely used method of identification and characterization of lectins. Lectins are commonly classified on the basis of their carbohydrate binding specificities.

Glycoconjugates are carbohydrate-rich molecules (e.g., glycoproteins and glycolipids) located both intracellularly and extracellularly and also at the cell surface which can be secretory or structural. Cell surface glycoconjugates usually undergo modification in association with certain cellular phenomena such as cell differentiation, adhesion, proliferation, morphological changes, functional modulation of cells, and under certain pathological conditions, such as neoplastic malignancy (Newman, 1979; Atkinson and Hakimi, 1980; Howard et al., 1981; Zieske and Bernstein, 1982).

3.2.1 Biochemistry and Common Properties of Lectins

Most lectins presently used in laboratories can be classified into five groups according to their preferential binding to D-pyranose sugars: (a) glucose/mannose group; (b) N-acetylglucosamine group, (c) galactose or N-acetylgalactosamine group; (d) L-fucose group, and (e) sialic acid or neuraminic acid group.

Monosaccharide specificity of lectins

Since most lectins react with the terminal, non-reducing sugars of glycoproteins or glycolipids components of cell membrane, testing for specificity usually includes common cell surface oligosaccharides and / or sugars accounting for blood group specificity or forming the internal sequence of carbohydrate chains: D-galactose (Gal), N-acetylgalactosamine (GalNAc), glucose (Glc), N-acetylglucosamine (GlcNAc), mannose (Man), L-fucose (Fuc), and sialic acid (NeuAc). The outcome of testing is conventionally expressed in relative terms with some sugars being very potent, others being less efficient inhibitors, while still others are completely inefficient. It is, however, noteworthy that the binding of many lectins can be effectively inhibited by more than one sugar and that the most potent inhibition is sometimes achieved by complex or derivatized synthetic sugars that are not necessarily found in the cell membrane of red blood cells.

Many lectins with the same nominal monosaccharide specificity show different affinity to the same cell or tissue structure in histochemical preparations (Alroy et al, 1984; Virtanen et al., 1986). However, it would be impractical to use an extensive series of inhibitors, and the monosaccharide inhibition data for lectin specificity are only a rough guide from which one could estimate their reactivity in tissues. Thus, biochemical data obtained from lectin histochemistry on tissue sections should be interpreted with caution. Binding of certain lectins to tissues that cannot be completely inhibited with the specific monosaccharide, or the differential binding of several lectins of the same "biochemical specificity" to the same structure, is always an indication that these lectins are reacting with complex carbohydrates. Although the reactive glycoconjugates most likely contain the specific monosaccharides, the actual binding sites are more complex than one could anticipate from extrapolating biochemical data obtained by hapten inhibition. Lectins of the same nominal specificity may recognize different surface features of that oligosaccharide and this may influence their histochemical properties (Petryniak and Goldstein, 1986).

Inhibition of lectin binding

Lectins of defined specificity are often inhibited by more than one sugars. Many lectins tolerate some variation at the C-2 position, very little variation at the C-3, and essentially do not tolerate any variation on the C-4 position of the pyranose ring (Goldstein and Poretz, 1986). Structural similarities or the differences on these three carbon atoms account for their affinity to glucose residues of the mannose binding lectins such as Con A, LCA and PSA *(Full names and details of lectins refer to Table 3.1)*. Most GalNAc binding lectins, like MPA, GS-I or BPA also react with Gal residues (Debray et al., 1981; Wu, 1984). In practical terms, unless extensive biochemical or enzymatic controls are included, mannose-binding lectins are not reliable probes for distinguishing Man from Glc residues, and GalNAc residues cannot be reliably distinguished from Gal residues. However, Man/Glc specific lectins generally react differently as compared to the Gal/GalNAc binding lectins.

Derivatized Lectins

The conjugation of biotin, agarose or fluorescein isothiocyanate to lectins usually do not alter the binding properties of lectins to carbohydrate ligands (McCoy, 1986). However, it was noticed that some forms of derivatization, such as the addition of acetyl or succinyl residues, may alter the binding properties of some lectins but have no effect on others (Monsigny et al., 1980). Derivatized lectins may have some advantages over their native forms. For example, succinylated-Con A is more stable and gives more consistent results than the native lectin Con A, although under properly controlled conditions, the histochemical results are comparable (Lee and Damjanov, 1984).

3.2.2 Lectins as Probes for Biological Markers Detection

Lectins have been extensively used to study a number of cellular phenomena in non-mineralizing tissues such as change of cell surface glycoconjugates during epidermal differentiation (Zieske and Bernstein, 1982; Nemanic et al., 1983); localization of specific saccharides in the extracellular matrix and basement membranes of adult tissues (reviewed by Spicer et al., 1983b). Lectins have also been used to study the complex carbohydrates or mucus of tracheal and alimentary secretions (Hotta et al., 1982, Bradley and Spicer, 1983; Spicer et al., 1983a; Thomopoulos et al., 1983; Fischer et al., 1984) as well as to analyze changes in glycoconjugates accompanied with diseases, including neoplastic transformation (reviewed by Coggi et al., 1983). In cultured cells, lectins have been widely used as histochemical or differential markers to carbohydrate moiety of glycoconjugates in the process of synthesis and secretion of glycoproteins and their incorporation into the plasma membrane (Tartakoff and Vassalli, 1983; Griffiths et al., 1982, 1983).

Since complex glycoproteins are reliable biochemical markers, lectins capable of recognizing these glycoproteins have been extensively used as biochemical and histochemical probes in cell biology and pathology (Lis and Sharon, 1986; Alroy et al., 1984). At the subcellular level, lectins have been used to study the glycoconjugates of various cytoplasmic organelles, including endoplasmic reticulum as well as Golgi apparatus, and micro-domains of cell membranes (Roth, 1983a; Tartakoff and Vassalli, 1983). At the tissue level, lectins have been used as specific probes to localize and characterize various cell types (Holthofer et al., 1982; Nakagawa et al., 1986) as well as cells at various stages of differentiation or maturation (Coapman and Cooper, 1986; Virtanen et al., 1986). At the level of organ histology, lectins have been used as probes for delineation of functional and anatomic regions (Faraggiana et al., 1982; Hennigar, 1987) or micro-environments (Woody et al., 1986). Lectins have also been used for sequential and comparative studies to provide clues regarding ontogeny and phylogeny of various organs and tissues (Farnum and Wilsman, 1986; Holthofer and Virtanen, 1987). Functional

changes have also been explored (Walker, 1984; Whyte and Allen, 1985), as well as changes associated with or leading to malignant transformation (Shiba et al., 1984), metastasis (Kahn and Baumal, 1985) and tumor cell heterogeneity (Virtanen et al., 1985).

Identification of specific cell types

Due to differential glycosylation of cell membranes and cytoplasmic components in highly specialized cells, lectins may be used as reagents for identification and study of various cell types. This approach is particularly useful to study the carbohydrate histochemistry of organs composed of several cell types, such as liver and pituitary gland. From many studies of this type, several examples bearing relevance for pathologists will be presented in order to illustrate the use of lectins as probes and markers for certain cell types.

The anterior pituitary gland is composed of several types of distinct hormone secreting cells, some of which produce glycosylated hormones such as prolactin which is secreted by the chromophilic cells is a glycoprotein. Nakagawa and co-workers (1986) have shown that pituitary cells differ in their lectin binding affinities for various lectins. In human pituitary, the corticotrophes are stained selectively with fucose- binding lectins (LTA) whereas thyrotrophils could be demonstrated selectively by Con A, a glucose and mannose binding lectin. Lectin binding pattern in human pituitary differs from those of the pituitaries of dogs and rats, obviously due to species differences. However, the lectin binding pattern of a specific organ or cell type is species specific, indicating the complexity of glycosylation. Thus lectin histochemistry are widely applied to distinguish different cell types.

Developmental Changes

Differentiation and maturation of various cell types in developing tissues as well as in tissues undergoing constant renewal can be efficiently studied by lectin histochemistry. The distinct lectin binding patterns of human skin have been related to the keratinization of epidermis (Virtanen et al., 1986). In the adult skin, the basal cell layer is outlined by DBA, while the suprabasal cell layers are reactive to SBA and HPA, UEA-I reacts with upper cell layers of epidermis (Zieske and Bernstein, 1982). In contrast, fetal epidermis dose not show such compartmentalization revealed by lectin. Lectin histochemistry shows that the maturing epidermal cells change not only their shape and keratin cytoskeleton but also their cell surface glycoconjugates.

In a detailed study of fetal human kidney, Holthofer and Virtanen (1987) showed that the appearance of lectin binding pattern is related to the developmental stages which is parallel with the morphologic changes of the nephron and the appearance of nephron specific antigens. It is therefore proposed that changing patterns of lectin binding reflect the sequential loss of embryonic glycoconjugates, growth related modification of cell components, functional maturation of cells, and the expression of adult forms of glycoconjugates.

Immunochemical studies on early mouse embryos have already indicated the importance of cell surface glycoconjugates in embryogenesis and have shown that these surface macromolecules appear and disappear from the cell surface in a developmentally regulated manner (Fox et al., 1981; Hamada et al., 1983). Lectin histochemical studies supplement these immunochemical data and provide additional evidence for complex glycosylation of stage specific antigens (Sato et al., 1986). Thus, stage specific antigen 1 (SSEA-1) which is known to contain a galactose and fucose component, appears on the surface of 16 cell-stage mouse embryos. Receptors for the fucose binding lectin (LTA) appear at the same developmental stage, indicating that the embryonic glycoprotein is fucosylated at that particular stage of development. A fucosyltransferase, presumably mediating

this reaction, has also been isolated (Muramatsu et al., 1986). It is of interest that UEA-I, another fucose-binding lectin, reacts quite differently with embryos as compared to LTA, suggesting that the conformational changes which occurs during embryogenesis determine the reactivity of the fucose residues. Similar observations on the different binding patterns of lectins with similar sugar specificity have been noticed with some GalNAc reacting lectins (Sato and Muramatsu, 1985). This may highlight subtle differences and changes that are not detectable with monoclonal antibodies and other immunochemical reagents.

Functional Changes

Glycosylation of the epithelial cells lining the uterine cavity is under the influence of hormones of pregnancy. It was shown that these changes can be detected by lectins in both murine and human endometria (Lee et al., 1983; 1985). The endometrial epithelial cells from pregnant mice express RCA-I, MPA, and BPA binding sites in their cell surface but absent in the non-pregnant mouse uterus. On the other hand, epithelial cells of non-pregnant uterus binds the Fuc-specific lectin UEA-I, in contrast to the pregnant uteri which do not express receptors for this lectin. In other words, pregnancy hormones induce the expression of Gal/GalNAc-rich glycoconjugates and a concomitant loss of Fuc-residues. Although there are no explanations for the changes in glycosylation during pregnancy, it is obvious that lectin binding patterns reflect the hormonally altered function of these cells.

Extracellular matrix

Farnum and Wilsman (1986) have used lectin histochemistry to study the intracellular and extracellular glycoconjugates in the growth-plate cartilage of Yucatan swine and have shown that lectin histochemistry may provide additional information to morphologic and biochemical. Ohnoa and colleagues (Ohnoa et al.,

1986) also showed that lectins may be used for the analysis of extracellular matrix of rat tracheal cartilage.

Systematic lectin histochemical studies of extracellular matrix have not been performed as yet. Fragmentary data from the literature indicate that basement membranes found in various tissue differ in their reactivity with lectins. Thus, GS-I seems to be a good marker for basement membranes in murine tissues (Peters and Goldstein, 1979). It was demonstrated that GS-I reacts with some, but not all, basement membranes (Wu et al., 1983). Peters and Goldstein (1979) reported that GS-I does not react with the Bowman's capsule or basement membranes of mouse heart and skeletal muscle.

Yashioka et al. (1987) have tested several lectins and shown that only RCA-I may inhibit the binding of antirenal basement membrane antibody to the kidney. This indirectly illustrates the heterogenesities of lectin binding sites on renal basement membranes. RCA-I reacts also with other membranes and could thus be used for lectin histochemical studies of human extracellular matrix.

3.2.3 Lectin Binding Specificity of Chondrocytes

Cartilage extracellular matrix is an intricate molecular network composed of type II collagen fibers, proteoglycan aggregates and glycoproteins. Biosynthesis of extracellular matrix by chondrocytes involves extensive processing of nascent proteins. In particular, glycosylation reactions are known to occur during intracellular elaboration of the major secretory products which are type II procollagen (Olsen, 1981) and chondroitin sulphate proteoglycan (CSPG) (Roden, 1980). Also link proteins, the stabilizers in the aggregation of proteoglycans to hyaluronan are glycosylated (Roughley et al., 1982). Fragmentary information about the cellular biosynthesis of some of these molecules has mostly come from biochemical studies. For instance, a model has been proposed for the addition of glycosaminoglycan and

oligosaccharide chains to the nascent core protein of CSPG (Geetha-Habib et al., 1984; Kimura et al., 1984). However there are not many studies concerning the coordinate synthesis and glycosylation of different extracellular components (Vertel et al., 1985 a & b).

WGA and PNA have been shown to be able to bind the matrix proteoglycans of bovine hyaline cartilage (Della Rocca et al., 1995). WGA, and SBA can bind to the extracellular matrix of rabbit temporomandibular joint cartilage (Griffiths et al., 1992). SBA, PNA and UEA also show distinct staining pattern in fibrillated patellar articular cartilage (Schunke et al., 1985).

3.2.4 Lectin Binding Specificity of Growth Plate

Chondrocytes from both hyaline and elastic cartilage share the common characteristics of producing an extracellular matrix composed of type II collagen and proteoglycans which are capable of forming high molecular weight complex with hyaluronic acid. However, in contrast to other cartilage, growth-plate is transitory; and individual chondrocytes within the growth plate exist for only a brief time in comparison with the whole growth plate. Thus, growth-plate cartilage as a temporal structure is superimposed upon the spatial organization of the tissue. It has been hypothesized that the chondrocytes themselves initiate and control the complex events of matrix calcification and vascularization through their control of the composition and organization of the extracellular matrix (Boskey, 1981; Wuthier, 1982). Recent studies have focused upon unique phenotypic markers to distinguish growth-plate cartilage from other cartilage (Schmid and Linsenmayer, 1983; Gibson et al., 1984; Poole et al., 1984; Sussman et al., 1984). In addition, the possibility that growth-plate chondrocytes change their phenotypic expression dependent on the stage of their cellular maturation has been explored, and matrix glycoproteins and collagens unique to specific cellular zones have been described (Schmid and Conrad, 1982; Poole et al., 1984; Remington et al., 1984; Kielty et al., 1985)

In the swine distal radial growth plate, PNA, RCA-I and SBA show positive reaction on the peri-cellular matrix of all zones while WGA stains only the peri-cellular matrix of resting zone (Farnum and Wilsman, 1988). Intracellular cytoplasmic binding to Con-A, LCA and LTA are also demonstrated. Nagano (1992) has reported a electron microscopic cytochemical study on lectin binding sites in the physeal cartilage-plate of rabbit tibia. Con A binding was found in the rough endoplasmic reticulum as well as the cis side of the Golgi apparatus. The Con A binding becomes increasingly abundant from the younger proliferating stage, through the maturing stage to the stage of hypertrophy, and diminished abruptly at the stage of calcification. WGA and PNA are also observed on the cis side of Golgi apparatus. PNA binding is also found within the nucleus.

In the present study, lectin-binding histochemistry is used to analyze intracellular and intercellular glycoconjugates which are characteristic of the phenotype of growth-plate chondrocytes *in situ*.

3.3 METHODS

3.3.1 Materials

Streptavidin-peroxidase, competing simple sugars, diaminobenzidine tetrahydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Biotin-labeled lectins were obtained from E.Y. Laboratories (San Mateo, CA) and Sigma Chemical Co.

3.3.2 Tissue Preparation

Pigs aged between 4 - 5 weeks (about 10 kg) of both sexes were used in this study. After the animal was sacrificed by injecting 10 ml of 2.5% pentobarbital directly into its heart, physeal and costal growth plates were dissected and fixed in 4% paraformaldehyde in phosphate buffer saline (PBS). The specimen were then decalcified with 0.4% EDTA in phosphate buffered saline for 4 weeks (Bancroft and Stevens, 1990). After decalcification, the tissue was dehydrated in graded series of ethanols, cleared in xylene and embedded in paraffin. 5μm-thick paraffin sections were cut.

3.3.3 Lectin Histochemistry

The sections were dewaxed, hydrated, and then treated according to the following procedures (Chan and Wong, 1991; Hsu and Raine, 1982; Jones and Stoddart, 1986). The sections were first incubated in 0.3 - 0.5 % (v/v) hydrogen peroxidase in absolute methanol for 10 - 20 min. to block the endogenous peroxidase activity. After washing in 0.05 M Tris buffered saline (TBS, pH 7.6) for 15 min, the sections were then incubated with a variety of biotinylated lectins at concentration of 10 μg/ml in 0.05 M TBS, pH 7.6, containing 0.1 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$ for 1 hr at room temperature. The lectins used in this study are listed in Table 3.1, which also includes their major sugar specificities and corresponding competing sugars (Goldstein and Poretz, 1986). After washing with 0.05 M TBS, sections were incubated with 2 μg/ml peroxidase-labeled streptavidin in 0.05 M TBS for 1 - 2 hr. The lectin binding sites were then visualized by the glucose oxidase-

diaminobenzidine(DBA)-nickel procedure (Shu et al, 1988; Chan and Choi, 1995). In brief, the sections were preincubated with ammonium nickel sulfate-DBA solution (0.4g ammonium nickel sulfate, 20 mg β-D-glucose, 4.0 mg ammonium chloride and 5.0 mg DAB in 10ml 0.1M acetate buffer pH 6.0) for 5 - 10 min and then glucose oxidase solution (0.5mg per 2.0ml distilled water; Sigma, Type VII) was added. The section was incubated with mixture solution for 1 to 5 min. The reaction intensity which is a dark purple/black color was checked every minute. The reaction was stopped by incubating the sections in 0.1M sodium acetate, pH 6.0. After washing the sections with distilled water for 10 min, some sections were counter-stained briefly with Mayer's hematoxylin. Then the sections were dehydrated and mounted for microscopic examination. Control sections were prepared by either omitting lectin solutions or pre-incubating the lectin solutions with their corresponding specific competing sugar solutions at a concentration of 0.2M for 30 - 60 min. before applying them to the tissue sections.

Table 3.1 Summary of Lectins Used in This Study

Lectins	*Source*	Binding Specificity	Known binding Oligosaccharide
1. Glucose / Mannose			
GNA	*Calanthus nivails*	α-Man	Man(α1,3)Man
S- Con A	*Canavalia ensiformis*	α-Man, α-Glc, α-GlcNAc	Branched Mannoses
LCA	*Lens culinaris*	α-Man, α-Glc, α-GlcNAc	Branched Mannoses with α-Fucose as determinant
PSA	*Pisum sativum*	α-Man, α-Glc, α-GlcNAc	Branched Mannoses with α-Fucose as determinant
2. N-acetylglucosamine			
PWA	*Phytolacca americana*	β-GlcNAc	- - -
DSA	*Datura stramonium*	β-GlcNAc	GlcNAc(β1,4)GlcNAc oligomers; Gal(b1,4)GlcNAc
STA	*Solanum tuberosum*	β-GlcNAc	GlcNAc(β1,4)GlcNAc oligomers
UEA II	*Ulex europaeus*	β-GlcNAc	L-Fuc(α1.2)Gal(β1,4)-GlcNAc
UDA	*Urica diolca*	β-GlcNAc	- - -
WGA	*Triticum vulgaris*	β-GlcNAc	GlcNAc(β1,4)GlcNAc
GS-II	*Griffonia simplicifolia*	α– & β-GlcNAc	- - -
3. N-acetygalactosamine or galactose			
HAA	*Helix aspersa*	α-GalNAc	- - -
HPA	*Helix pomatia*	α-GalNAc	- - -
VVA	*Vicia villosa*	α-GalNAc	GalNAc(α1,3)Gal
DBA	*Dolichos biflorus*	α-GalNAc	
GS-IB	*Griffonia simplicifolia*	α-GalNAc & α-Gal	- - -
Jacalin	*Artocarpus integrifolia*	α-GalNAc & α-Gal	Gal(β1,3)GalNAc
MPA	*Maclura pomifera*	α-GalNAc & α-Gal	Gal(β1,3)GalNAc
BPA	*Bauhinia purpurea*	α- & β- GalNAc	- - -
SBA	*Glycine max*	α- & β- GalNAc	GalNAc(α1,3)Gal
WFA	*Wistaria floribunda*	α- & β- GalNAc	GalNAc(α1,6)Gal
ECA-II	*Erythrina cristagalli*	α-, β- GalNAc & α-, β-Gal	Gal(β1.4)GlcNAc
PNA	*Arachis hypogaea*	β-Gal	GalNAc(α1,3)Gal
RCA-I	*Ricinus communis I*	β-Gal	Lactose
RCA-II	*Ricinus communis II*	β-Gal & β-GalNAc	Lactose
SJA	*Sophora japonica*	β-GalNAc	GalNAc(β1,6)Gal
4. L-fucose			
Lotus A	*Lotus tetragonolobus*	α-Fucose	Fuc(α1,2)Gal(β1,4)[Fuc(α1,30]GlcNAc
UEA-I	*Ulex europaeus*	α-Fucose	Fuc(α1,2)Gal(β1,4)-GlcNAc
5. Sialic acid			
SNA	*Sambucus nigra*	Sialic Acid & β-Gal	NANA(a2,6)Gal/GalNAc
MAA	*Maackia amurensis*	Sialic Acid	NANA(a2,6)Gal
LFA	*Limax flavus*	Sialic Acid	- - -
LPA	*Limulus polyphemus*	Sialic Acid	- - -
6. Oligosaccharide			
PHA-L	*Phaselous vulgaris*	Oligosaccharide	Gal(β1,4)GlcNAc(β1,2)-Man(α1,6)..
PHA-E	*Phaselous vulgaris*	Oligosaccharide	Gal(β1,4)GlcNAc(β1,2)-Man(α1,6)..

Abbreviations: Man, mannose; Glc, glucose; GlcNAc, N-acetyglucosamine; GalNAc, N-acetylgalactosamine; Gal, galactose, Fuc, L-Fucose.

3.4 RESULTS

The glycoconjugates of the porcine costal and physeal growth plate were demonstrated by lectin histochemistry using the avidin-biotin-glucose oxidase-DAB-nickel method (Shu et al.,1988; Chan and Choi, 1995). The distribution and intensity of the lectin staining are summarized in Tables 3.2 and 3.3.

3.4.1 Definitions of cellular zones and matrix compartments

Although mammalian growth plates are similar in their basic microscopic structure, there are no standardized criteria for classification into different cellular zones (Brighton, 1978; Buckwalter, 1983; Buckwalter et al., 1985; Eggli et al., 1985). In the present study, cellular zones were defined by morphological criteria of cellular size, shape, and spatial relationships to other chondrocytes. The growth plate was defined as the cartilage between the secondary center of ossification and the metaphyseal bone. The reserve or resting zone was defined as that part of the growth plate in which chondrocytes occur singly or in pairs. On the physeal side, the reserve zone is adjacent to short columns of four to eight hypertrophic cells adjacent to the secondary center of ossification. These hypertrophic chondrocytes together with reserve zone chondrocytes collectively are the physeal component of articular-physeal complex cartilage (A-E complex) surrounding the secondary center of ossification (Farnum, 1985) (Figure 3.1). The proliferative cell zone was defined as that part of the growth plate where chondrocytes occur in columns of at least five cell per column. The hypertrophic cell zone is the distal region of the growth plate where the proximo-distal diameter of chondrocytes is greater than one-half of the transverse diameter (Reinholt et al., 1984; Buckwalter et al., 1985).

In the costal growth plate, the orientation of the zones is much organized (refer to Chapter 2 - Figure 2.1). The hypertrophic zone cartilage adjacent to the osteochondral junction and contains large sized chondrocytes. The proliferative

zone is characterized by the columns of chondrocytes. The region beyond proliferative zone and containing chondrocytes in single or in pair is defined as resting zone.

3.4.2 Glycosyl- and Mannosyl- Specific Lectins

Succinyl-Con A (S-Con A) is the active dimmer form which does not aggregate to form tetrameters (Con A) above pH 5.6. It is more stable and provides a more consistent result than Con A. The binding pattern was similar to that of Con A. S-Con A stained the cell membrane of proliferative and hypertrophic chondrocytes in both costal and physeal growth plates, and the cytoplasm of all chondrocytes showed positive reaction. Except the hypertrophic zone in the physeal growth plate cartilage, S-Con A stained the matrix of resting, proliferative, and hypertrophic zone of both types of growth plate cartilage (Figure 3.2).

The matrix of the proliferative zone cartilage in both costal and physeal growth plates was stained by GNA, while PSA stained only the cartilage matrix of resting and proliferative zones of the costal growth plate. LCA showed no reaction to the cytoplasm and membrane of the chondrocytes and cartilage matrix in the resting, proliferative as well as hypertrophic zones in both costal and physeal growth plates.

3.4.3 N-Acetylglucosaminyl- Specific Lectins

N-acetylglucosamine residues were absent in the costal growth plate as shown by its negative reaction to all GluNAc specific lectins tested, except WGA and GS-II which showed positive staining in the cytoplasm of resting chondrocytes. In the physeal growth plate, WGA stained the cytoplasm and membrane of resting chondrocytes. However, matrix of proliferative rather than resting zone cartilage was stained by WGA (Figure 3.3). DSA also showed positive reaction to matrix of proliferative zone.

3.4.4 N-Acetylgalactosaminyl- and Galactosyl- Specific Lectins

N-acetygalactosamine or galactose appeared to be the most abundant carbohydrate residues in the growth plate. The cytoplasm and cell membrane of chondrocytes as well as the surrounding matrix in various zones of the costal growth plate were strongly labeled by RCA-I. The reaction intensity appeared to be strongest at the hypertrophic chondrocytes. In the physeal growth plate, RCA-I reacted strongly with the cartilage matrix, especially in the proliferative zone. A moderate reactivity was noted in both the cytoplasm and membrane of hypertrophic chondrocytes. The membrane of resting chondrocytes was also intensely labeled (Figure 3.4).

The reactions to RCA-II were almost identical to RCA-I. The cytoplasm and cell membrane of chondrocytes as well as the surrounding matrix in various zones of costal growth plate were strongly labeled. The reaction intensity appeared to be strongest at the hypertrophic zone. In the physeal growth plate, RCA-I reacted strongly with the matrix, especially in the proliferative zone. A moderate reactivity was noted in both the cytoplasm and membrane of hypertrophic chondrocytes. The membrane of resting chondrocytes was also intensely labeled.

In costal growth plate, VVA stained the cytoplasm of the proliferative as well as hypertrophic chondrocytes, and the matrix of resting zone cartilage showed weak reaction. In physeal growth plate matrix, all zones showed positive reaction. Cytoplasm and membrane of resting chondrocytes were moderately stained. Membrane of proliferative chondrocytes was also stained (Figure 3.5).

DBA stained intensively with matrix in all zones of both type of growth plates, especially in the proliferative and hypertrophic zones. Cytoplasm and membrane of resting and hypertrophic chondrocytes in costal growth showed positive staining, while only the membrane of hypertrophic chondrocytes in physeal growth plate showed positive result (Figure 3.6).

The matrix of all zones in costal growth plate showed positive reaction to Jacalin. Jacalin stained also resting chondrocytes in the same type of growth plate. In physeal it stained only the matrix of proliferative zone cartilage.

WFA stained moderately the matrix of all cartilage zones in costal growth plate. A positive reaction was also seen in the matrix of proliferative and hypertrophic zone cartilage in physeal growth plate.

In costal growth plate, PNA stained the entire cytoplasm of all stage of chondrocytes. The matrix of different regions were stained, especially in the hypertrophic zone. However, PNA reacted with only the proliferative zone matrix in physeal growth plate.

Only the matrix of proliferative zone cartilage in physeal growth plate was stained by HAA, while the matrix of resting zone cartilage in physeal growth plate was weakly and unevenly labeled by ECA-II.

Matrix of proliferative zone cartilage in physeal growth plate showed weak to negative staining to SJA while no reaction was seen in the other intracellular and extracellular components.

HPA, GS-IB_4, MPA, BPA and SBA had showed no reaction to cytoplasm, membrane of the chondrocytes and matrix of all the resting, proliferative as well as hypertrophic zone cartilage in both costal and physeal growth plates.

3.4.5 L-fucosyl- Specific Lectins

The matrix of the three cartilage zones in physeal growth plate showed positive labeling to LTA. The rest of the growth plate resulted in a negative reaction (Figure 3.6).

3.4.6 Sialic Acid Specific Lectins

The cytoplasm of proliferative and hypertrophic chondrocytes in costal growth plate showed positive labeling to SNA. The matrix of the proliferative and hypertrophic zone cartilage of physeal growth plate were also stained (Figure 3.7).

MAA, LFA and LPA had no reaction to cytoplasm, membrane and matrix of all the resting, proliferative as well as hypertrophic zone cartilage in both costal and physeal growth plates.

3.4.7 Oligosaccharide Specific Lectins

In physeal growth plate, PHA-L reacted moderately with the matrix in resting and proliferative zones whereas PHA-L exhibited variable reactivities in hypertrophic zone. However, only the matrix in hypertrophic zone of costal growth plate showed positive labeling.

Except the resting zone in costal growth plate, PHA-E stained the entire matrix in various regions of both types of growth plate. The proliferative chondrocyte membrane of physeal growth plate also gave positive reactions (Figure 3.8).

Table 3.2 Lectin Binding Pattern of Costal Growth Plate

	Cytoplasm			Membrane			Matrix		
	Rest	Prolif	Hyper	Rest	Prolif	Hyper	Rest	Prolif	Hyper
Glucose / Mannose									
GNA	-	-	-	-	-	-	-	+	-
S-Con A	+	+	+	-	+	+	+	+	+
LCA	-	-	-	-	-	-	-	-	-
PSA	-	-	-	-	-	-	+	+	-
N-acetylglucosamine									
PWA	-	-	-	-	-	-	-	-	-
DSA	-	-	-	-	-	-	-	-	-
STA	-	-	-	-	-	-	-	-	-
UEA-II	-	-	-	-	-	-	-	-	-
UDA	-	-	-	-	-	-	-	-	-
WGA	+	-	-	+	-	-	-	-	-
GS-II	+	-	-	-	-	-	-	-	-
N-acetygalactosamine/galactose									
HAA	-	-	-	-	-	-	-	-	-
Jacalin	+	-	-	-	-	-	+	+	+
GS-IB	-	-	-	-	-	-	-	-	-
HPA	-	-	-	-	-	-	-	-	-
MPA	-	-	-	-	-	-	-	-	-
VVA	-	+	+	-	-	-	+	-	-
SBA	-	-	-	-	-	-	-	-	-
PNA	+	+	+	-	-	-	+	+	+ +
RCA-I	+	+	+	+	+	+	+	+	+ +
RCA-II	+	+	+	+	+	+	+	+	+ +
ECA-II	-	-	-	-	-	-	-	-	-
DBA	+	-	+	+	-	+	+	+ +	+ +
WFA	-	-	-	-	-	-	+	+	+
BPA	-	-	-	-	-	-	-	-	-
SJA	-	-	-	-	-	-	-	-	-
L-fucose									
LTA	-	-	-	-	-	-	-	-	-
UEA-I	-	-	-	-	-	-	-	-	-
Sialicate									
SNA	-	+	+	-	-	-	-	-	-
MAA	-	-	-	-	-	-	-	-	-
LFA	-	-	-	-	-	-	-	-	-
LPA	-	-	-	-	-	-	-	-	-
Oligosaccharide									
PHA-L	-	-	-	-	-	-	-	-	+
PHA-E	-	-	-	-	-	-	-	+	+

The intensity of lectin staining was graded as "-" : negative; "+": moderate binding; "++": strong binding. Abbreviations used: Rest=resting; Prolif=proliferative; Hyper=hypertrophic zone cartilage.

Table 3.3 Lectin Binding Pattern of Physeal Growth Plate

		Cytoplasm			Membrane			Matrix		
		Rest	Prolif	Hyper	Rest	Prolif	Hyper	Rest	Prolif	Hyper
Glucose / Mannose										
	GNA	-	-	-	-	-	-	-	+	-
	S-Con A	+	+	+	-	+	+	+	++	-
	LCA	-	-	-	-	-	-	-	-	-
	PSA	-	-	-	-	-	-	-	-	-
N-acetylglucosamine										
	PWA	-	-	-	-	-	-	-	-	-
	DSA	-	-	-	-	-	-	-	+	-
	STA	-	-	-	-	-	-	-	-	-
	UEA-II	-	-	-	-	-	-	-	-	-
	UDA	-	-	-	-	-	-	-	-	-
	WGA	+	-	-	+	-	-	-	+	-
	GS-II	-	-	-	-	-	-	-	-	-
N-acetygalactosamine/galactose										
	HAA	-	-	-	-	-	-	-	+	-
	Jacalin	-	-	-	-	-	-	-	+	-
	GS-IB	-	-	-	-	-	-	-	-	-
	HPA	-	-	-	-	-	-	-	-	-
	MPA	-	-	-	-	-	-	-	-	-
	VVA	+	-	-	+	+	-	+	+	+
	SBA	-	-	-	-	-	-	-	-	-
	PNA	-	-	-	-	-	-	-	+	-
	RCA-I	-	-	+	++	-	+	+	++	+
	RCA-II	-	-	+	++	-	+	+	+	+
	ECA-II	-	-	-	-	-	-	+	-	-
	DBA	-	-	-	-	-	+	+	++	++
	WFA	-	-	-	-	-	-	-	+	+
	BPA	-	-	-	-	-	-	-	-	-
	SJA	-	-	-	-	-	-	-	+	-
L-fucose										
	LTA	-	-	-	-	-	-	+	+	+
	UEA-I	-	-	-	-	-	-	-	-	-
Sialicate										
	SNA	-	-	-	-	-	-	-	+	+
	MAA	-	-	-	-	-	-	-	-	-
	LFA	-	-	-	-	-	-	-	-	-
	LPA	-	-	-	-	-	-	-	-	-
Oligosaccharide										
	PHA-L	-	-	-	-	-	-	+	+	+/-
	PHA-E	-	-	-	-	+	-	+	+	+

The intensity of lectin staining was graded as "-" : negative; "+": moderate binding; "++": strong binding. Abbreviations used: Rest=resting; Prolif=proliferative; Hyper=hypertrophic zone cartilage.

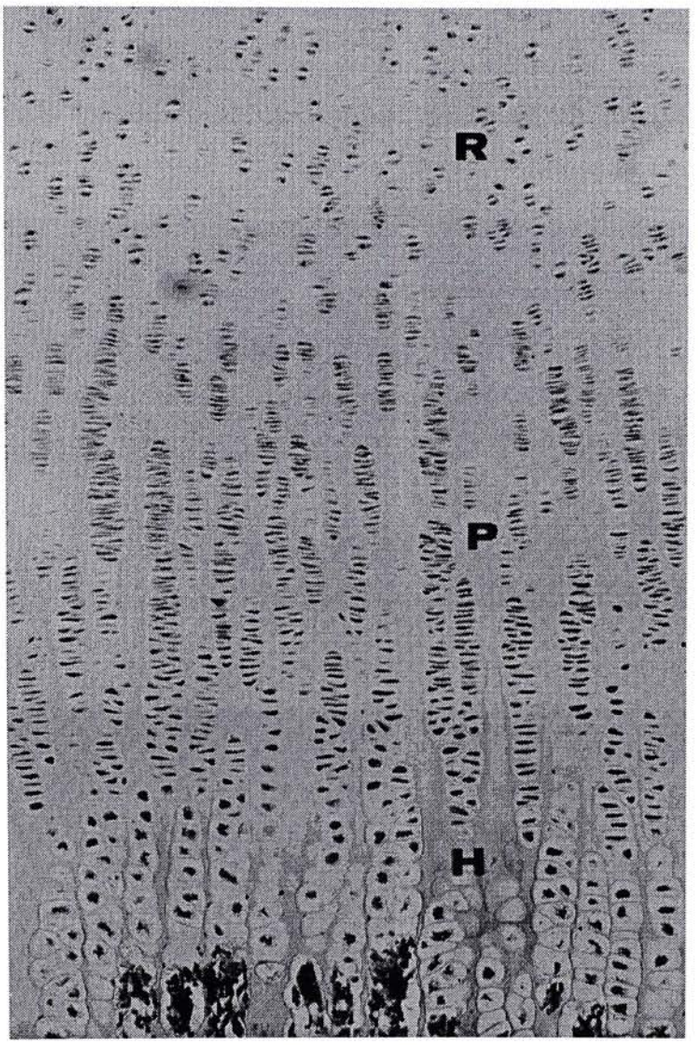

Figure 3.1 Longitudinal sections of porcine physeal growth plate. The cellular size, shape, and spatial relationships of resting (R), proliferative (P) and hypetrophic zone are illustrated. (H&E, 10x)

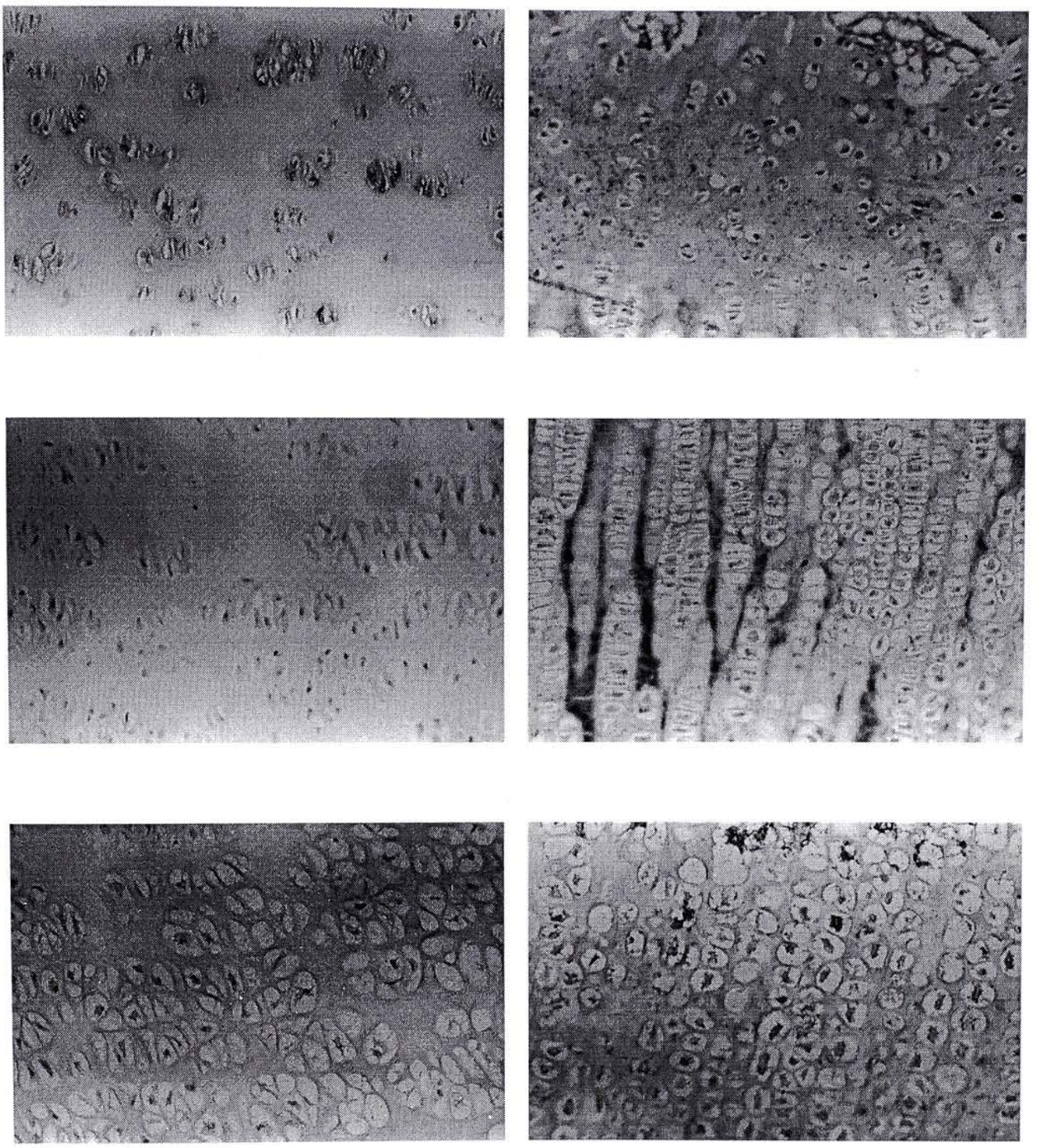

Figure 3.2 S-ConA staining patterns of resting (R), proliferative (P) and hypertrophic (H) zone cartilages in porcine costal and physeal growth plates. (20x)

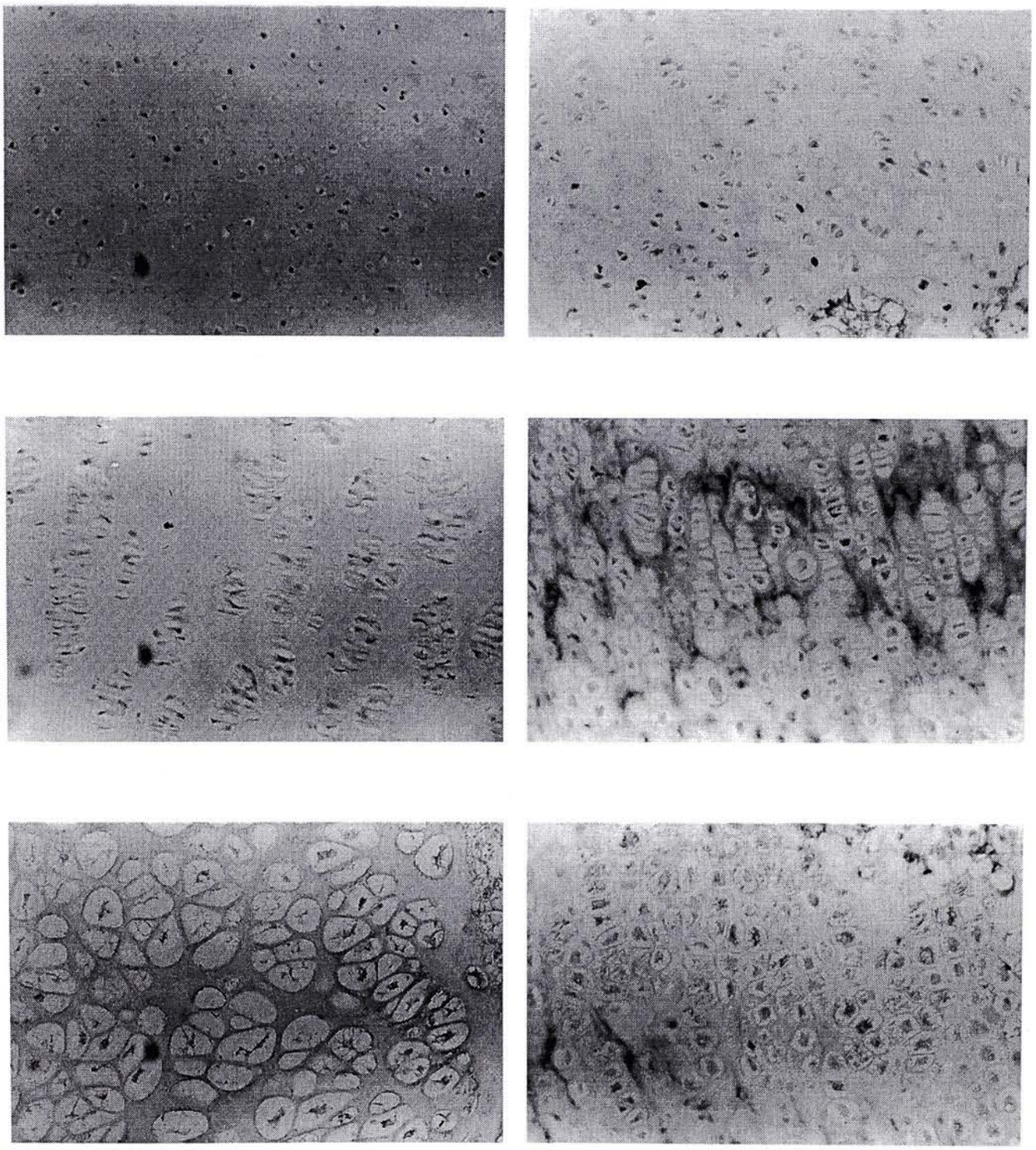

Figure 3.3 WGA staining patterns of resting (R), proliferative (P) and hypertrophic (H) zone cartilages in porcine costal and physeal growth plates. (20x)

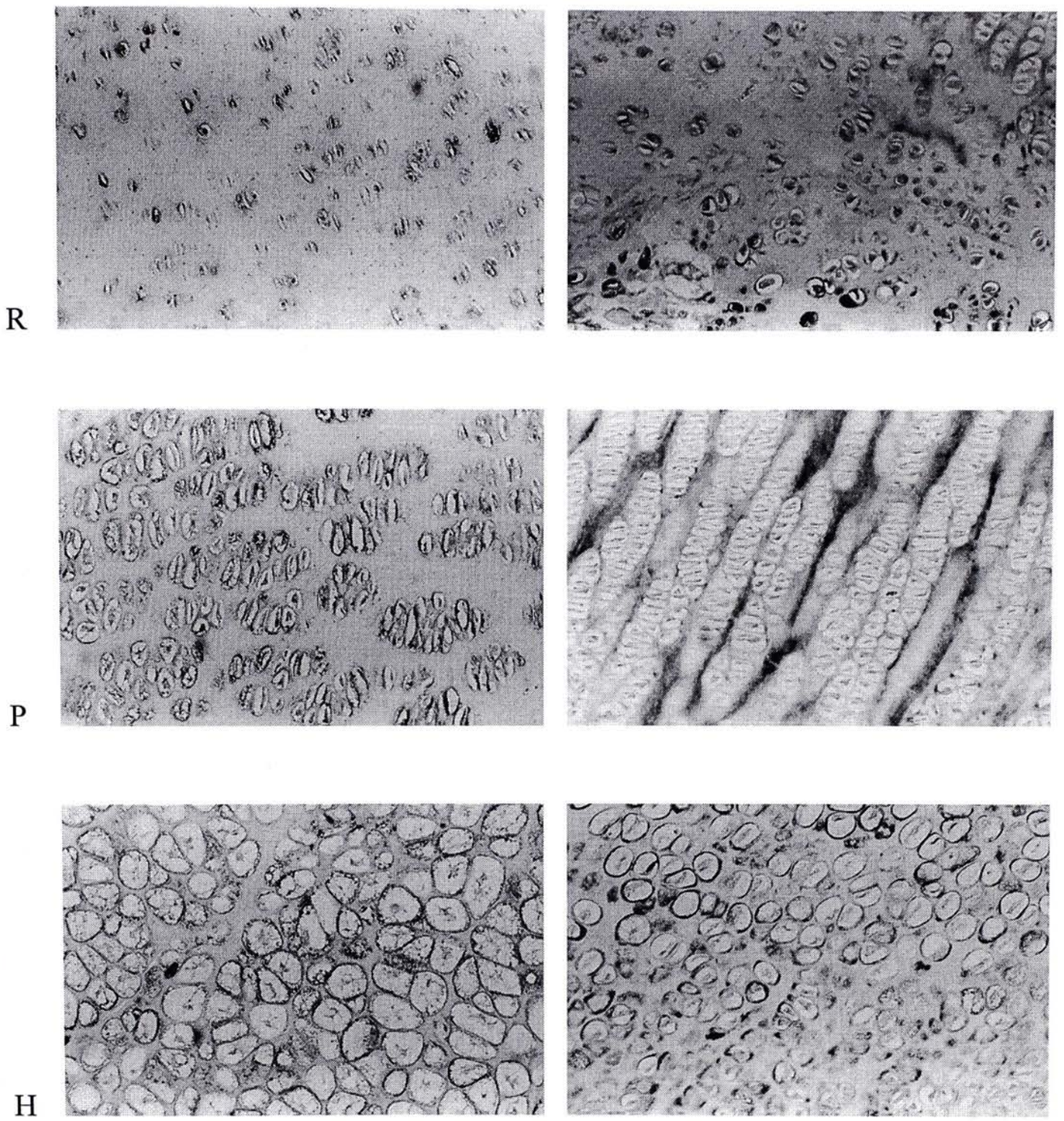

Figure 3.4 RCA-I staining patterns of resting (R), proliferative (P) and hypertrophic (H) zone cartilages in porcine costal and physeal growth plates. (20x)

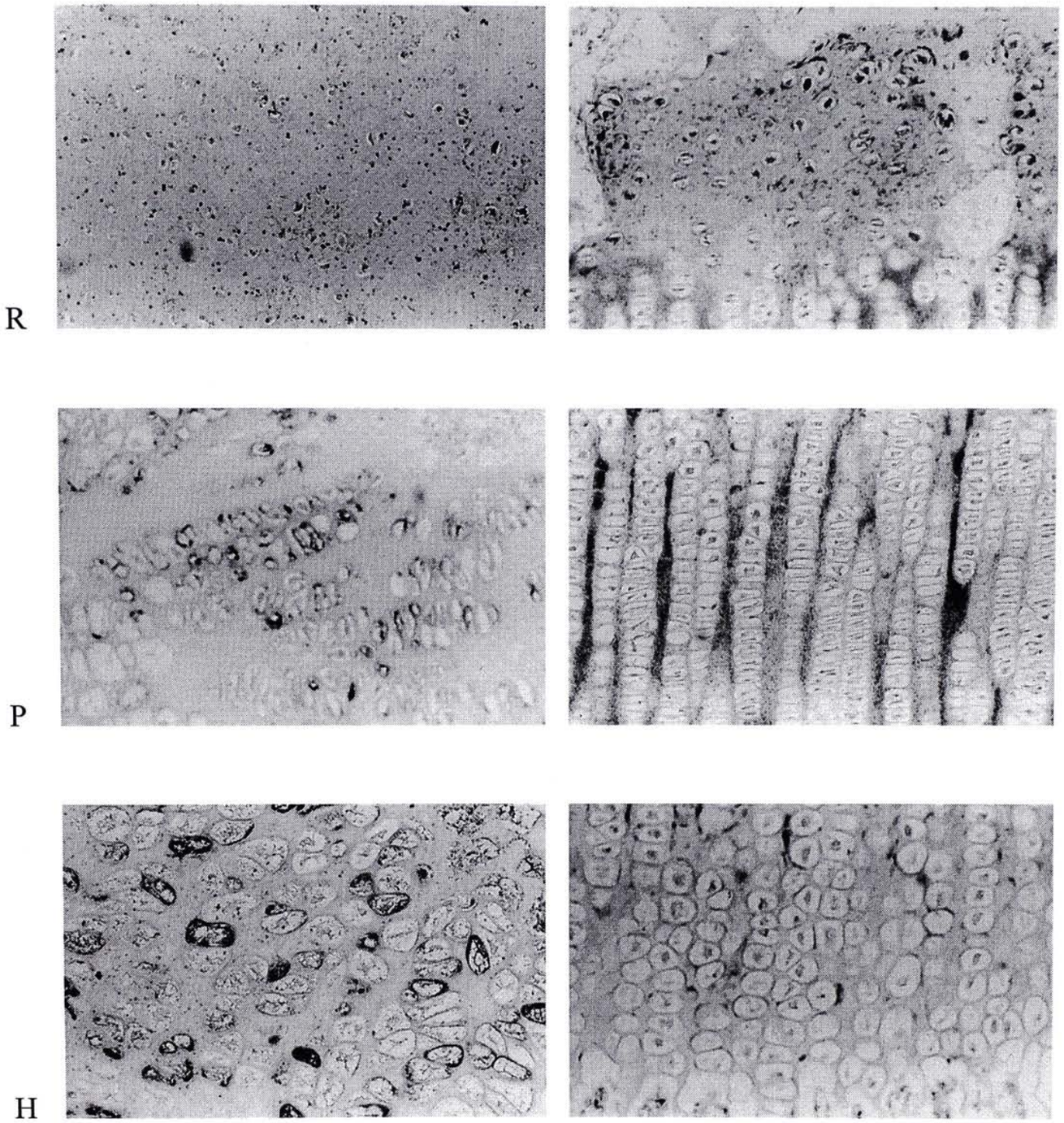

Figure 3.5 VVA staining patterns of resting (R), proliferative (P) and hypertrophic (H) zone cartilages in porcine costal and physeal growth plates. (20x)

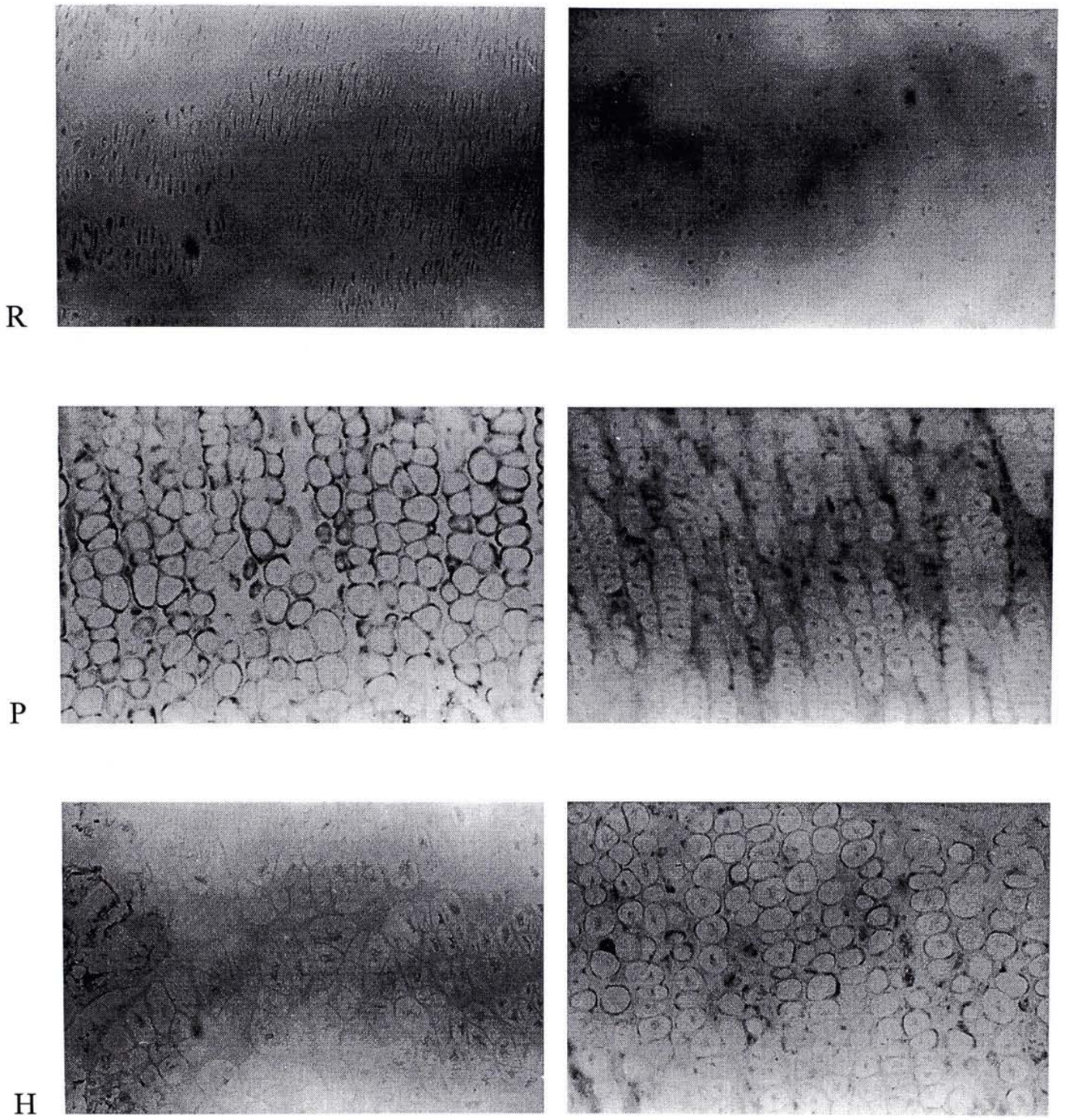

Figure 3.6 LTA staining patterns of resting (R), proliferative (P) and hypertrophic (H) zone cartilages in porcine costal and physeal growth plates. (20x)

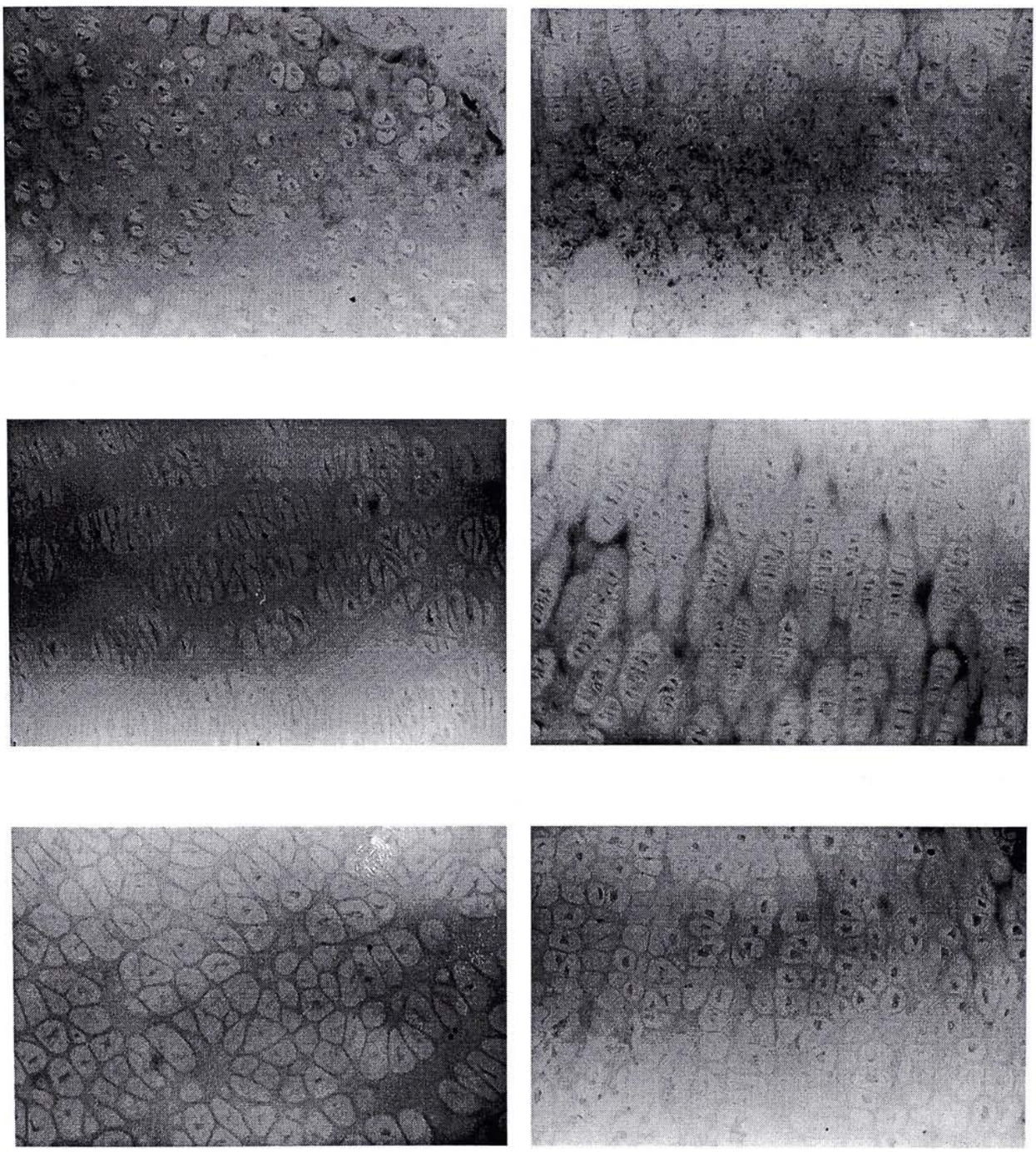

Figure 3.7 SNA staining patterns of resting (R), proliferative (P) and hypertrophic (H) zone cartilages in porcine costal and physeal growth plates. (20x)

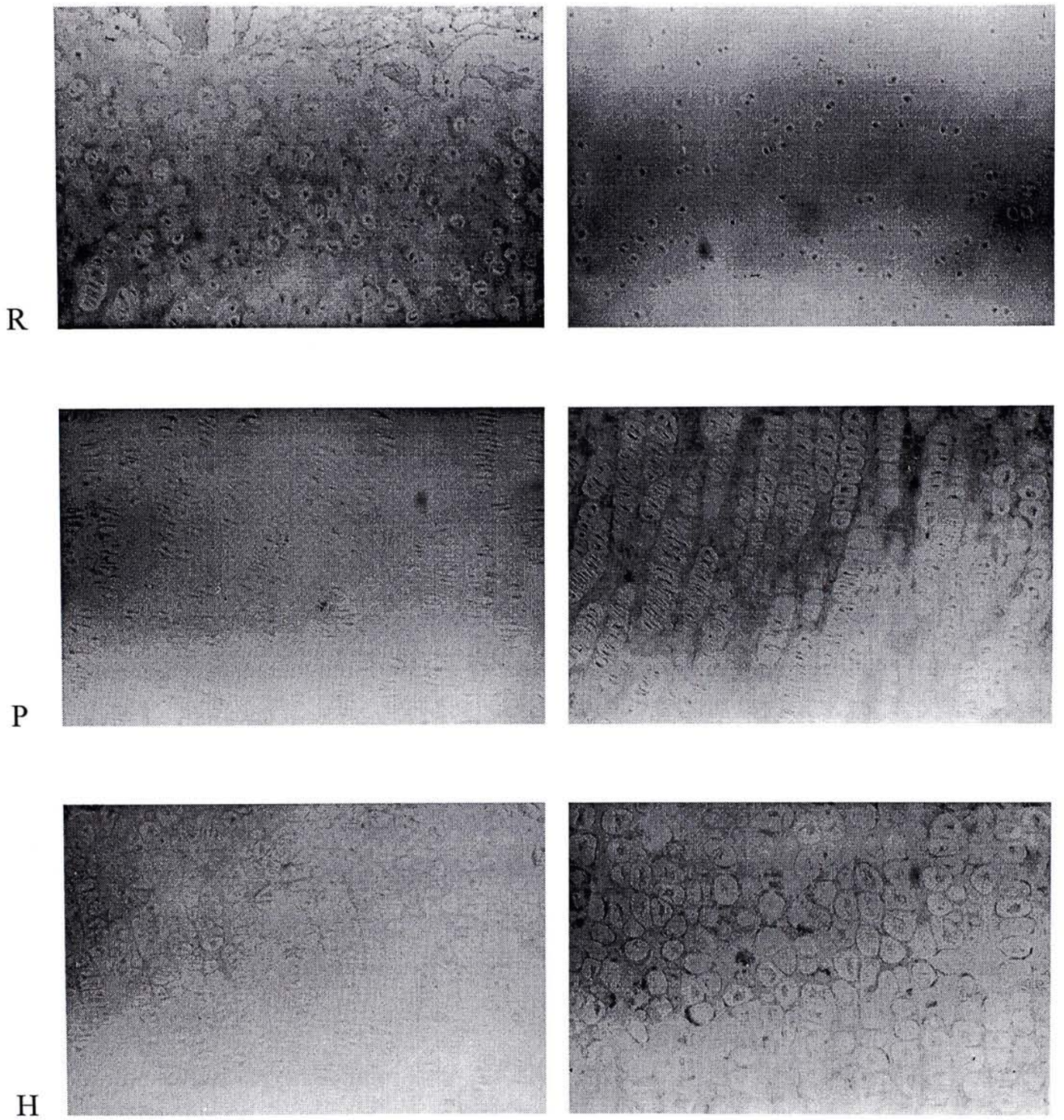

Figure 3.8 PHE-E staining patterns of resting (R), proliferative (P) and hypertrophic (H) zone cartilages in porcine costal and physeal growth plates. (20x)

3.5 DISCUSSION

Chondrocytes from both hyaline and elastic cartilage share the common characteristics of producing an extracellular matrix composed of type II collagen and proteoglycans which form high-molecular weight aggregates with hyaluronic acid. However, in contrast to other cartilage, growth-plate cartilage is transitory; and individual chondrocytes within the growth plate exist for only a brief time in comparison with the whole growth plate. Thus, growth-plate cartilage as a temporal structure is superimposed upon the spatial organization of the tissue. It has been hypothesized that the chondrocytes themselves initiate and control the complex events of matrix calcification and vascularization in the process of endochondral ossification through their control of the composition and organization of the extracellular matrix (Boskey, 1981; Wuthier, 1982). Recent studies have focused upon unique phenotypic markers to distinguish growth-plate cartilage from other cartilage (Schmid and Linsenmayer, 1983; Gibson et al., 1984; Poole et al., 1984; Sussman et al., 1984). In addition, during maturation, the changes of the phenotypic expression of the growth-plate chondrocytes, as well as the matrix glycoproteins and collagens of specific zones have been described (Schmid and Conrad, 1982; Poole et al., 1984; Remington et al., 1984; Kielty et al., 1985; Schmid and Linsenmayer, 1985).

In the distal hypertrophic zone of the physeal growth plate cartilage, matrix calcification, vascularization, and ossification occur. It is hypothesized that the changes in interactions of extracellular matrix components in different zones of the epiphysis control the process of endochondral ossification. These changes involve converting an uncalcifiable matrix of the proliferative zone to a calcificable matrix of the hypertrophic zone, and a matrix that resists vascular invasion proximally to a matrix that is invaded by metaphyseal vasculature distally. The control mechanisms for these complex interactions probably are multiple (Boskey, 1981; Wuthier, 1982).

Biochemical analysis of regions of the growth plate have been hindered by the difficulty of obtaining samples of sufficient quantity, the heterogeneity of macromolecular components, and the presence of endogenous proteases. The validity of morphological analysis is dependent upon the ability to preserve the extracellular matrix components and histochemical markers simultaneously. Despite these potential limitations, evidence has shown that the hypertrophy of chondrocytes is accompanied with changes in the cartilage matrix components, particularly the collagens and proteoglycans (Poole et al, 1982a; Schmid and Conrad, 1982; Buckwalter, 1983; Shepard and Mitchell, 1985).

Although collagen and proteoglycans are the principal macromolecules of growth plate cartilage, interactions of chondrocytes with less abundant matrix components, such as non-collagenous structural glycoproteins, may be also significant in the changes which accompany the maturation of chondrocytes. Results from *in vitro* studies suggest that pericellular glycoproteins may be involved in such diverse phenomena as maintenance of a characteristic cell shape and state of differentiation, cellular migration, and the organization of the extracellular matrix (Vaheri and Alitalo, 1981; Olden et al., 1985). Studies on the non-collagenous glycoproteins in the growth plate cartilage are limited, and the results on their distribution and significance are still conflicting (Weiss and Reddi, 1980; 1981; Hevitt et al., 1982; Poole et al., 1982a&b). Therefore, it is necessary to further analyse the glycoproteins in the growth plate by histochemical and immuno-histochemical techniques in order to understand better the changes of the molecular events during the endochrondral ossification (Poole et al., 1982; Buckwalter, 1983).

In the present study, the intracellular components and extracellular matrix of porcine growth plate cartilage *in situ* were analyzed by lectin histochemistry. Lectins purified from plants and animals are proteins or glycoproteins which are specific to individual saccharides or oligosaccharide. Because of these sugar binding specificities, lectins when conjugated to different histochemical markers have been widely used to investigate the glycoconjugates in cells and tissues. This approach is especially

useful for the study of the composition and organization of the glycoconjugates of growth plate cartilage.

Interpretation of lectin-binding patterns

Some precautions are considered when analyzing the lectin-histochemistry. First, it is not possible to identify specific macromolecules from the results of lectin-histochemistry since the same sugar residues or oligosaccharides may express in more than one macromolecules. Lectins can bind to saccharide sequences both on the link protein and on the monomer core protein of the proteoglycan aggregate (Nilsson et al., 1982; Thonar et al., 1983). The linkage structure between keratan sulfate and the core protein of proteoglycans is related to that of O-linked oligosaccharides, and essentially identical oligosaccharide side chains are found on a variety of structural glycoproteins of the cartilage matrix which are not involved directly in proteoglycan aggregate formation. Binding of WGA and succinyl-WGA to GlcNAc residues requires non-substitution at C3 position (Allen et al., 1973; Monsigny et al., 1980), and so potentially these lectins can react with the repeating disaccharide unit of keratan sulfate, but not to hyaluronic acid (Toda et al., 1981; Vertel et al., 1985 a & b).

Second, the specificities of lectins established by *in vitro* analysis may not be consistent with *in situ* lectin binding patterns as the histochemical binding can be affected by a number of factors including fixation and accessibility of the potential lectin binding site (Ochoa, 1981). Therefore, positive results in the present study are interpreted to represent the existence of accessible lectin-binding sites under the conditions of these experiments, and at the level of resolution of light microscopy. Negative results do not imply the absence of lectin-binding sites.

Third, in numerous studies it has been shown that lectins with similar sugar-binding affinities may exhibit different binding pattern (Debray et al., 1981; Holthofer, 1983; Schulte and Spicer, 1983 a & c). Mucous secretions, which had been thought previously to be homogeneous, have been found to be heterogeneous in their glycoconjugates as shown by the lectin histochemistry. (Bradley and Spicer, 1983;

Schulte and Spicer, 1983b; Fischer et al., 1984). In many cases, however, the biochemical identity of the heterogeneity has yet to be elucidated.

Lectin Binding Patterns of Growth Plates

From the results of this study, S-Con A bound to the membranes of proliferative and hypertrophic chondrocytes. The cytoplasm of chondrocytes at all zones were also stained. These findings suggest that α-mannose may be essential to growth plate chondrocytes. This observation is similar to that of Nagano (1992) on the physeal cartilage plate of rabbit tibia. He demonstrated that most Con-A binding sites were found in the rough endoplasmic reticulum (RER), while some bindings were found on the cis side of the Golgi apparatus. It becomes increasingly abundant from the younger proliferative stage, through the maturing stage to the stage of hypertrophy.

The Con A binding to intracellular glycogen has been reported in the keratinocytes of the skin (Hyun et al., 1984) and this binding cannot be removed by pretreatment with amylase. Pollesello et al. (1991) demonstrated by ^{31}P-NMR that resting and hypertrophic chondrocytes of pig scapulas growth plate depend on glycolysis for their energy production. Thus, the intracellular binding with Con A in the chondrocytes probably represents stored glycogen.

All the four tested glucose and mannose binding lectins showed positive reactions to the matrix of proliferative zone cartilage in both costal and physeal growth plate. This may indicates that α-mannose is highly incorporated into the cartilage matrix in the proliferative zone.

N-acetylglucosamine residues were not found in the growth plate cartilage matrix as shown by its negative reaction to most GluNAc specific lectin tested. However, WGA and GS-II can bind to cytoplasm of resting chondrocytes. PWA and WGA weakly stained the matrix of proliferative zone cartilage.

Besides GlcNAc resides, WGA has been reported to react *in vitro* with sialic acid and its binding affinity to GlcNAc can be increased when clustered sialic acid residues

are present (Monsigny et al., 1980). Studies have shown that WGA binds *in vitro* to both high-affinity and low-affinity sites.

Glycoconjugates with sialic acid residues seems not expressed in resting zone cartilage as no positive reaction was observed after SNA staining. However, SNA stained the chondrocyte cytoplasm and matrix of proliferative and hypertrophic zones. This indicates that sialic acid residues are expressed in the later stage of maturation.

In the costal growth plate cartilage matrix, glycoconjugates rich in N-acetygalactosamine and galactose residues appeared to be prominent as shown by its positive staining with Jacalin, PNA, RCA-I, RCA-II DBA and WFA to the hypertrophic zone matrix. This indicated that N-acetygalactosamine and galactose containing glycoconjugates in the matrix became increasingly abundant from resting zone, through the maturing zone to the zone of hypertrophy. This result is consistent with the previous findings by Farnum and Wilsman (1988), whom demonstrated that PNA and RCA-I show positive reaction on the peri-cellular matrix of all zones in the swine distal radial growth plate.

β-galactose was expressed in growth plate chondrocytes, since PNA, RCA-I and RCA-II showed positive labeling to cytoplasm and membrane of chondrocytes in all zones. Similar results were also observed in the physeal growth plate. The positive PNA staining in the chondrocyte cytoplasm are likely restricted to the Golgi region. Nagano (1990) has demonstrated the PNA binding sites in the cis side of Golgi apparatus in the chondrocytes of the rabbit physeal growth plate.

The previous studies have shown that the distribution of the RCA-I-binding glycoconjugates in hypertrophic chondrocytes was identical to the distribution of fibronectin in the rabbit (Weiss and Reddi, 1981). Then Font and Aubrey (1983) further proved that fibronectin was an RCA-I binding glycoprotein. Therefore, the RCA-I binding glycoconjugates demonstrated in the growth plate cartilage in this study may represent the fibronectin.

The positive staining of the fucose-binding LTA with the matrix of physeal growth plate is also observed by Farnum and Wilsman (1988). In contrast, another fucose-binding lectin, UEA-I showed negative staining to the cartilage matrix. LTA has affinity for difucosyl moieties, as compared to UEA-I which is specific to monofucosyl oligosaccharides. These two lectins also differ in affinity for the glycosidic linkage of the branch fucose (Pereira et al., 1978). This discrepancy in their sugar specificities could probably explain their different staining patterns in the present case. These different binding patterns between UEA-I and LTA have also been reported previously in kidney and salivary glands (Laden et al., 1984). In addition, glycoconjugates with L-fucose can only be found in matrix of physeal growth plate but not the costal growth plate. It demonstrated that expression of glycoconjugates in growth plate cartilage could be site-specific.

Oligosaccharide binding lectins, PHA-L and PHA-E, stained the cartilage matrix only but were non-reactive to the chondrocytes, suggesting some oligosaccharides containing glycoconjugates are present in the matrix.

Site Specificity of Lectin Binding Pattern

A slight discrepancy in lectin binding patterns was observed in both costal and physeal growth plate cartilage. Some lectins (e.g. GS-II) showed similar binding patterns in both costal and physeal growth plate cartilage, while other lectins (e.g. SNA) bound to the chondrocytes of either cartilage. It indicates that expression of glycoconjugates in the cartilage may be site specific.

Differential Expression of Lectin Binding Glycoconjugates

The expression of some glycoconjugates of the differentiating chondrocytes appeared to be stage-dependent during the process of endochondral ossification. For example, S-Con A binding glycoconjugates were expressed during the maturation process of the chondrocytes. Moreover, the chondrocytes in the resting zone were positive to

VVA, while its staining disappeared after the resting. However, the expression of other glycoconjugates (e.g. RCA-I and RCA-II binding) appeared to be independent of the maturation process of the chondrocytes.

Differentiation Markers for the Growth Plate Chondrocytes

The results of the lectin histochemical study showed that the differentiating chondrocytes in the porcine physeal growth plate express characteristic lectin binding patterns (summarized in Table 3.2 & 3.3). In the physeal growth plate, WGA bound specifically to the resting chondrocytes, while DBA and PHA-E bound to the proliferative and hypertrophic zone chondrocytes. These lectins could be used as the differentiation markers for the growth plate chondrocytes.

Future Applications for the Differential Lectin Binding Pattern for Growth Plate Chondrocytes

For the application of the findings obtained in this study, separation and identification of chondrocytes in various maturation stages could be possible according to their different membrane glycoconjugate expression (e.g. WGA and DBA in Table 3.4) by approaches such as lectin affinity chromatography and flow cytometry using fluorescein-labeled lectin probes. For example if we release the chondrocytes from costal growth plate by enzyme digestion (method refer to the material & method in chapter one) and stain the chondrocytes in various maturation stages with FITC-labeled WGA and Cye 5- labeled DBA. The fluorescein-labeled chondrocyte mixture can be applied to the flow cytometer. Then the three chondrocyte subpopulations, named resting (double labeled), proliferative (non-labeled) and hypertrophic (DBA labeled) will be obtained. The separated chondrocyte subpopulations could be used for further biochemical and molecular characterization. It should provide us with better understanding of the endochondral ossification process.

Table 3.4. Example for Identification of Various Porcine Growth Plate Subpopulation by Binding Specificity of WGA and DBA

	WGA	DBA
Costal GP Chondrocyte		
Resting	+	+
Proliferative	-	-
Hypertrophic	-	+
Physeal GP Chondrocyte		
Resting	+	-
Proliferative	-	-
Hypertrophic	-	+

Chapter Four

Intra- & Extra-Cellular Free Calcium Activities of Porcine Growth Plate Chondrocytes at Various Stages of Maturation

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4.1 AIMS OF STUDY

The intracellular ionized calcium concentration ($[Ca^{2+}]_i$) has been demonstrated in many cells to play a critical role in the regulation of cellular metabolism. In the growth plate chondrocytes, the content of intracellular free calcium appears to increase during the processes of cellular maturation (from resting stage, proliferative stage to hypertrophic stage) and matrix mineralization (Iannotti & Brighton, 1989). The physiologic significance of the accumulation of large amounts of intracellular free calcium $[Ca^{2+}]_i$ in growth plate chondrocytes has not been determined but has been postulated to play a role in matrix mineralization and regulation of cellular metabolism. The aim of this study is to establish a model for *in situ* monitoring of intracellular and extracellular Ca^{2+} from each zone of the mammalian growth plate. The development of such *in situ* Ca^{2+} monitoring model enables us to investigate the effect of various modulators, such as TGF-α, TGF-β and PTH on the $[Ca^{2+}]_i$ among chondrocytes at different maturational stages during endochondral ossification.

4.2 LITERATURE REVIEW

Endochondral ossification involves a series of progressive stages of cartilage development ultimately leading to mineralization of the extracellular matrix. Although it is generally acknowledged that growth plate chondrocytes are vital to the mineralization process, some investigators suggest that these cells are primarily involved in producing an extracellular matrix capable of inducing mineral deposition. Nevertheless, there is a growing awareness that the cells may be directly involved in the acquisition and processing of Ca^{2+} and inorganic phosphate (Pi) before the appearance of the initial crystalline mineral in the extracellular domain. However, to date, relatively few studies have directly examined electrolyte metabolism by cells of mineralizing tissues (Brighton and Hunt, 1976; D'Andrea et al., 1990; Dziak et al., 1988; Gunter et al., 1990; Iannotti and Brighton, 1989; Shapiro and Lee, 1995).

4.2.1 Physiological Role of Calcium in Endochondral Calcification

Past work established that matrix vesicles are primary initiators of extracellular mineral deposition in endochondral calcification. Recently, there are studies showing that cellular metabolism of Ca^{2+} and inorganic phosphate (Pi), and cellular interaction with the matrix are involved in the formation of calcifiable matrix vesicles. Chondrocytes in growth plate cartilage are envisioned to induce the formation of calcifiable matrix vesicles. These studies indicate that growth plate chondrocytes actively acquire Ca^{2+}, concentrate it to the cell periphery and exfoliate it as Ca^{2+}-rich matrix vesicles. Data from direct chemical analysis and ^{31}P-NMR studies on freshly isolated cells show that growth plate chondrocytes are depleted of ATP and have elevated cytosolic Pi, a prerequisite to form Ca^{2+}-acidic phospholipid (APL)-Pi complex-primed matrix vesicles (Geisow and Walker, 1986). Chondrocyte cell membrane processes from which matrix vesicles arise have been found to be

tightly linked to the cartilage-specific extracellular matrix collagens and proteoglycans. Annexins V and Vl, APL-dependent Ca^{2+}-binding proteins that form Ca^{2+} channels in chondrocytes and matrix vesicles membranes, also bind to the matrix collagens and may serve as mechano-transducers in growth plate cartilage, gating Ca^{2+} entrance into the cells and matrix vesicles (Fernandez et al., 1988b; Genge et al., 1992). This interaction between the extracellular matrix and chondrocytes appears to facilitate Ca^{2+} loading of chondrocytes, formation of Ca^{2+} and Pi-primed matrix vesicles and rapid induction of mineralization in growth plate cartilage.

4.2.2 Difficulties in Studying Cellular Electrolytes During Endochondral Calcification in Growth Plate

Several factors have contributed to the lack of study of cellular metabolism of electrolytes during the mineralization process. A major factor has been the previous difficulty in measuring the levels of Ca^{2+}, Pi, and other electrolytes in intact living cells. Fortunately, with the recent advances in the development of sensitive, fluorescent chemical probes to measure intracellular Ca^{2+} ($[Ca^{2+}]i$) (Tsien et al. 1984, Tsien and Tsien 1990) and of ^{31}P nuclear magnetic resonance (NMR) for Pi (Burt et al. 1976, Pollesello et al., 1991), it has become possible to measure the levels of intracellular electrolytes in living cells *in vitro* under conditions in which calcification is in progress.

A second barrier has been the lack of knowledge of the proteins involved in transport of mineral ions through cellular membranes in growth plate chondrocytes. However, with recent progress in characterizing various ion-pumps and channels in cells generally (Adams et al 1989, Carafoli 1987, Langer 1992, Somlyo and Himpens 1989) and the recent cloning and sequencing (Fernandez et al. 1988a, Genge et al 1992), as well as determining the three-dimensional structure (Huber et al 1990) of what appears to be the major Ca^{2+} ion-channel protein in matrix vesicles, and

presumably chondrocytes (Genge et al. 1989, Genge et al. 1990), it has become possible to study in detail the transport of Ca^{2+}, Pi and other ions through lipid bilayer membranes (Montessuit et al. 1991, Pollard and Rojas 1988, Rojas et al 1990).

4.2.3 Calcium and Phosphate Metabolism by Growth Plate Chondrocytes

One of the fundamental questions pertaining to the induction of mineral deposition in tissues concerns whether cellular metabolism of Ca^{2+} and Pi is directly involved in this process. There is now considerable morphological and biochemical evidence in endochondral calcification that growth plate chondrocytes acquire substantial amounts of Ca^{2+} and Pi before the onset of extracellular mineralization (Brighton and Hunt, 1976; Gunter et al., 1990; Iannotti and Brighton, 1989; Shapiro and Lee, 1975). Growth plate chondrocytes produce matrix vesicles (Bonucci, 1970; Hale and Wuthier, 1987) that contain large amounts of Ca^{2+} and Pi (Arsenault et al., 1988; Wuthier and Gore, 1977) and mediate the induction of extracellular mineralization (Ali, 1976; Anderson, 1969; Bonucci, 1970). Although the mechanism of matrix vesicles mineralization is not fully understood, there is strong evidence that matrix vesicles derive from the plasma membrane (PM) of growth plate chondrocytes by blabbing of vesicles from cell processes (Bonucci, 1970; Hale and Wuthier, 1987). However, what causes growth plate cells to acquire high levels of Ca^{2+} and Pi, how they handle the elevated levels of these ions and whether the Ca^{2+} and Pi are directly transferred to the vesicles during matrix vesicles formation will be areas of active study in the future.

4.2.4 Interaction Between Chondrocytes and the Extracellular Matrix

Various lines of evidence indicate that the outer chondrocyte membrane interacts directly with extracellular matrix proteins, and this interaction contributes to matrix vesicles formation. Although earlier electron microscopic evidence pointed to an

attachment between cell processes and the extracellular matrix (Arsenault et al., 1988, Hunziker et al. 1984), recent biochemical data have detailed specific interactions between chondrocyte membrane proteins and cartilage-specific collagens (Fernandez et al., 1988b; Wu et al., l991a) and proteoglycans (Wu et al., l991b).

Of special significance is the observation that both type II and X collagen bind tightly to annexins V and VI (Wu et al. l991a) members of the recently discovered class of membrane-associated, APL-dependent Ca^{2+}-binding proteins (Geisow and Walker 1986). As noted previously, annexin V (anchorin CII, Fernandez et al. 1988a), one of the dominant proteins in matrix vesicles (Genge et al. 1989, Genge et al. 1990, Genge et al. 1992), exhibits Ca^{2+} channel activity when inserted into phospholipid bilayers (Rojas et aL 1990). Zn^{2+}, which blocks Ca^{2+} uptake by matrix vesicles (Sauer et aL 1989), also blocks ion-channel activity of annexin V (Rojas et al., 1992).

Thus, the combination of collagen-binding and Ca^{2+} ion-channel properties make annexin V and annexin VI, a less abundant matrix vesicles protein with even tighter collagen-binding properties (Wu et al. l99la), promising candidates for stretch-activated Ca^{2+} ion channels (Wuthier 1992). Such properties would enable influx of Ca^{2+} into the cells to be coordinated with mechanical stress to the tissue (Watson 1991). The binding of the annexins to collagen and their Ca^{2+}-dependent binding to alkaline phosphatase also help to explain the formation of matrix vesicles (Hale and Wuthier 1987), which are enriched in APL (Wuthier 1975) and, as noted above, in Ca^{2+} and Pi (Arsenault et al 1988, Wuthier 1977). Thus, metabolism of Ca^{2+} and Pi, by growth plate chondrocytes, which now can be seen to be intimately involved in matrix vesicles formation, clearly are integral features of endochondral calcification. Therefore study of Ca^{2+} activity of chondrocyte should be performed *in situ*.

4.2.5 Cellular Effects of Ca^{2+} Depend on Its Cytosolic Level

Beside the specific Ca^{2+} ion channel in the membrane of growth plate chondrocytes and their matrix vesicles, just like many other cells, intracellular Ca^{2+} is an important second messenger and its concentration is controlled by complicated mechanisms. Most intracellular Ca^{2+} ions are sequestered in the mitochondria and endoplasmic reticulum (ER) or other cytoplasmic vesicles. The concentration of Ca^{2+} ions free in the cytosol usually is kept below 0.2μM. Ca^{2+}-ATPases pump cytosolic Ca^{2+} ions across the plasma membrane to the cell exterior or into the lumens of the endoplasmic reticulum or other intracellular vesicles that store Ca^{2+}. Localized increases in the cytosolic level of free Ca^{2+} is critical to its function as a second messenger. Local concentrations of Ca^{2+} ions can be monitored with fluorescence dyes; in large cells, different Ca^{2+} concentrations can actually be detected in specific regions of the cytosol.

A small cytosolic protein called calmodulin, which is ubiquitous in eukaryotic cells, mediates many cellular effects of Ca^{2+} ions. Ca^{2+}-calmodulin complex binds to and activates many enzymes. One well-studied enzyme that is activated by the Ca^{2+}-calmodulin complex is cAMP phosphodiesterase, which degrades cAMP and terminates its effects.

Small increases in the level of cytosolic Ca^{2+}, which are often mediated by a rise in inositol 1,4,5-triphosphate (IP_3), trigger many cellular responses. In secretory cells, such as the insulin-producing β cells in the pancreatic islets, a rise in Ca^{2+} triggers the exocytosis of secretory vesicles and the release of insulin. In smooth or striated muscle cells, a rise in Ca^{2+} triggers contraction; in both liver and muscle cells, an increase in Ca^{2+} activates the degradation of glycogen to glucose 1-phosphate (Berridge, 1987).

4.2.6 Inositol 1,4,5-Trisphosphate Causes the Release of Ca^{2+} Ions from the Endoplasmic Reticulum

Binding of many hormones and growth factors to their cell-surface receptors on liver, fat, and other cells induces an elevation in cytosolic Ca^{2+} even when Ca^{2+} ions are absent from the surrounding medium (Berridge, 1987). In this situation, Ca^{2+} is released into the cytosol from the ER and other intracellular vesicles. The mechanism by which a hormone-receptor signal on the cell surface is transduced to the ER became clear in the early 1980s, when it was shown that a rise in the level of cytosolic Ca^{2+} often is preceded by the hydrolysis of an unusual phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), one of several inositol phospholipids found in the cytosolic leaflet of the plasma membrane (Berrideg, 1993).

Hydrolysis of PIP_2 by the plasma-membrane enzyme phospholipase C (PLC) yields two important products: 1,2-diacylglycerol (DAG), which remains in the membrane, and inositol 1,4,5-trisphosphate (IP_3), which is water soluble. Binding of hormones to certain G protein-coupled seven-spanning receptors activates PLC. Thus hormone-induced stimulation of PLC activity and/or subsequent generation of IP_3 are mediated by G protein linked receptors and receptor tyrosine kinases.

Once formed hormone stimulation of a target cell, IP_3 diffuses to the ER surface, where it binds to a specific IP_3-sensitive Ca^{2+} channel. IP_3 binding induces opening of the channel allowing Ca^{2+} ions to exit from the ER into the cytosol. The overall sequence of events from binding of hormones to a G protein-linked receptor to Ca^{2+} release is depicted in Figure 4.1.

Within a second of its formation, most IP_3 is hydrolyzed to inositol 1,4-bisphosphate, a molecule that cannot induce release of Ca^{2+} ions from the ER. As a result, the release of Ca^{2+} ions terminates quickly unless more IP_3 is generated by action of phospholipase C.

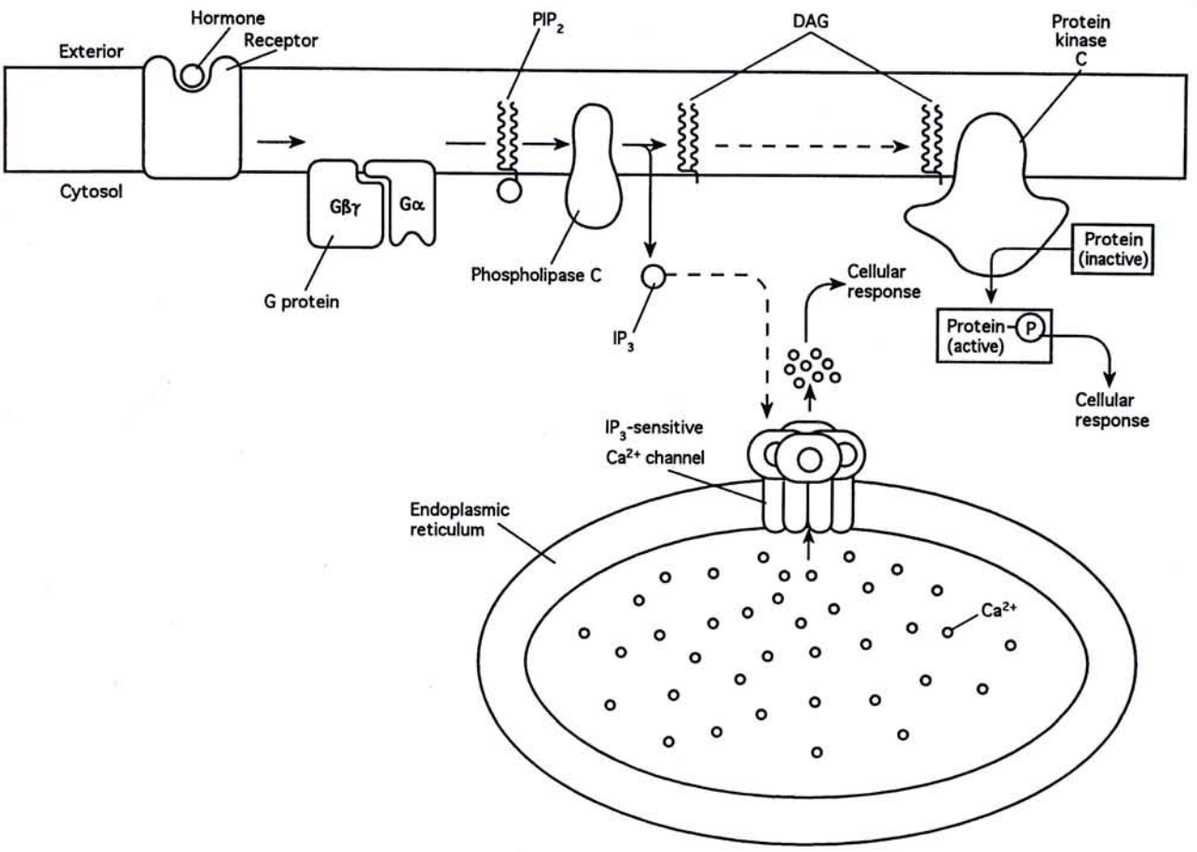

Figure 4.1 The inositol-lipid signaling pathway can be coupled to seven-spanning G protein-linked receptors, as illustrated here, and receptor tyrosine kinases. Binding of a hormone to its receptor triggers activation of the G protein (Go or Gq), which in turn activates phospholipase C by a mechanism analogous to activation of adenylate cyclase. Phospholipase C then cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). The IP_3 diffuses through the cytosol and interacts with IP_3-sensitive Ca^{2+} channels in the membrane of the endoplasmic reticulum, causing release of stored Ca^{2+} ions, which mediate various cellular responses. Protein kinase C is activated by Ca^{2+} and DAG, which remains in the membrane. The activated kinase then phosphorylates several cellular enzymes and receptors, thereby altering their activity [Adapted from Lodish et al., 1995, Molecular Cell Biology, pp 902]

Since continued hormone stimulation can deplete the store of Ca^{2+} ions in the ER within a few minutes, maintenance of elevated cytosolic Ca^{2+} levels requires transport of extracellular Ca^{2+} ions across the plasma membrane into the cell. Evidence suggests that Ca^{2+} entry into pancreatic cells is mediated by inositol 1,3,4,5-tetraphosphate, which is formed by phosphorylation of IP_3 by a specific kinase. In time, this inositol tetraphosphate is also hydrolyzed by a phosphatase, rendering it inactive. In other types of cells, the depletion of Ca^{2+} stores in the ER is thought to trigger the entry of extracellular Ca^{2+} ions by another mechanism not involving inositol phosphates (Berridge, 1993).

In addition, not all cells respond identically to IP_3. This differential sensitivity may reflect expression of different isoforms of the IP_3 receptor in the ER membrane and/ or variation in the Ca^{2+} content of the ER itself. Because of this variability, cells may exhibit very different responses to the same extracellular signal.

IP_3-sensitive Ca^{2+} channels are present in the ER membrane of many cell types. Although cytosolic Ca^{2+} cannot initiate opening of these Ca^{2+} channels in the absence of IP_3, low levels of Ca^{2+} ions potentiate the action of IP_3, resulting in greater release of stored Ca^{2+}. High concentrations of cytosolic Ca^{2+}, however, inhibit IP_3-induced release of Ca^{2+} from intracellular stores, the plasma membrane may also contain IP_3-regulated Ca^{2+} channels. The complex regulation of the IP_3 receptor in ER membranes can have unpredictable consequences. Frequently, rapid oscillations in the cytosolic Ca^{2+} level are seen when the IP_3 pathway in cells is stimulated (Berridge, 1987). Stimulation of hormone-secreting cells in the pituitary by LHRH causes rapid, repeated spikes in the cytosolic Ca^{2+} level; each spike is associated with a burst in secretion of luteinizing hormone (LH). One explanation for these Ca^{2+} spikes is that activation of phospholipase C and generation of IP_3 cause the release of a small amount of Ca^{2+} from the ER. The resultant increase in cytosolic Ca^{2+} causes, together with IP_3, more IP_3-sensitive Ca^{2+} channels to open and more Ca^{2+} to be released. This sets into motion an explosive, short-lived release of Ca^{2+} from the ER. Eventually, the rising level of cytosolic Ca^{2+} inhibits these

Ca^{2+} channels, no more Ca^{2+} is released, and the level of cytosolic Ca^{2+} drops quickly as Ca^{2+} is pumped back into the ER. Each cycle takes only a few seconds. The purpose of the fluctuations of Ca^{2+}, rather than a sustained rise in cytosolic Ca^{2+}, is not understood. One possibility is that a sustained rise in Ca^{2+} might be toxic to cells.

4.2.7 Release of Intracellular Ca^{2+} Stores is also Mediated by Ryanodine Receptors

In addition to IP_3-sensitive Ca^{2+} channels, muscle cells and neurons possess other Ca^{2+} channels called ryanodine receptors (RYRs), because of their sensitivity to the plant alkaloid ryanodine. In skeletal muscle cells, these receptors are located in the membrane of the sarcoplasmic reticulum and associate with the cytoplasmic domain of the dihydropyridine receptor, a voltage-sensing protein in the plasma membrane. A change in potential across the plasma membrane induces a conformational change in the RYR, so that Ca^{2+} ions are released from the sarcoplasmic reticulum into the cytosol. The ER membranes in cardiac muscle cells and neurons also contain RYRs, but they do not associate directly with a plasma-membrane protein. In these cells, depolarization of the plasma membrane leads to a small influx of extracellular Ca^{2+} ions through voltage-gated channels; binding of these Ca^{2+} ions to RYRs induces a burst of Ca^{2+} release from the ER or other intracellular stores.

4.2.8. Basic Theory of Confocal Microscopy

Confocal microscopy is particularly advantageous for 3D imaging of thick objects as a result of its optical sectioning property (Sheppard, 1987). It is widely used in the fluorescence mode for imaging biological objects of various types, but is also used in the bright-field reflection mode for imaging objects of different forms.

Light from a laser is focused by an objective lens onto the object, and the reflected, or fluorescent, light focused onto a photodetector via a beam-splitter. In the confocal microscope, a confocal aperture, or pinhole, is placed in front of the photomultiplier tube detector (Figure 4.2). An image is built up by scanning of the focused spot relative to the object, and is usually stored in a computer imaging system.

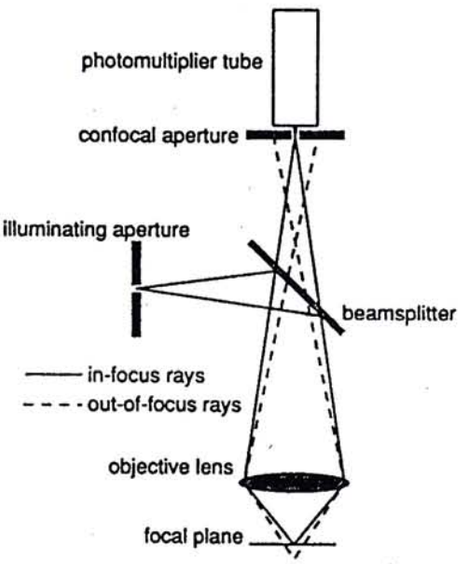

Figure 4.2 Schematic diagram of a confocal microscope. Light originating from points away from the focal plane is defocused at the confocal aperture and thus detected weakly. [Adapted from Mason W.T., Fluorescent and Luminescent Probes for Biological Activity, pp229]

Confocal microscopy is one particular imaging mode of the scanning optical microscope, which exhibits various advantages over conventional instruments as a result of the image being formed by a scanning technique.

Broadly, the advantages of scanning optical microscopy stem from two main properties. First is the fact that the image is measured in the form of an electronic signal, which allows a whole range of electronic image-processing techniques, both analogue and digital, to be employed. These include image-enhancement techniques such as frame-averaging, contrast enhancement, edge enhancement, and image subtraction to show changes or movement; image-restoration techniques for resolution enhancement and noise reduction, and image-analysis techniques such as feature recognition and cell sizing and counting.

Second is the property that imaging in a scanning microscope is achieved by illuminating the object with a finely focused light spot. This allows a number of novel optical imaging modes to be employed such as confocal imaging or differential phase contrast. It also introduces the possibility of imaging modes in which the incident light spot produces some related effect in the specimen which can be monitored to produce an image.

A particularly important class of such methods occurs when the wavelength of the detected radiation differs from that of the illumination. An advantage of using scanning techniques for such spectroscopic imaging is that imaging methods, performed with the incident radiation, are separated from wavelength selection and analysis of the emitted radiation, thus simplifying system design and resulting in superior performance. The detection system may also have greater sensitivity because it does not have to detect the noise image. This is of great advantage in fluorescence microscopy, which also results in the further advantage that the resolution is determined by the shorter incident wavelength, rather than the longer fluorescence wavelength.

Fluorescence, or luminescence, microscopy can give information concerning spatial variations in excitation states, binding energies, band structure, molecular configuration, structural defects, and the concentration of different atomic and molecular species.

4.3 METHODS

4.3.1 Longitudinal Costal Growth Plate Slices

Pigs between 4 - 5 weeks of age (about 10 kg) of both sexes were used in this study. After the animal was sacrificed by injecting 10 ml of 2.5% pentobarbital directly into its heart, the rib cage was aseptically dissected and cleaned until free from soft tissue. The growth plate cartilage was taken out at the osteochondral junctions of all the ribs. The growth plate block was then placed in a DMEM (Sigma, St. Louis, Missouri). About 300 μm thick longitudinal sections of growth plate were then prepared with vibrating microtone (Campdem Instruments Ltd., U.K.).

4.3.2 Intracellular Ca^{2+} Level Determination by Laser Scanning Confocal Microscopy

Ca^{2+} indictor loading

Determination of intracellular Ca^{2+} level by confocal laser scanning microscopy: Fluo-3, a Ca^{2+} fluorescent indicator compatible with laser excitation, was used to monitor changes in intracellular Ca^{2+} level. Growth plate sections were placed in a 3 cm diameter culture disk (Flocon, U.S.A.) and loaded with fluo-3/AM (6-8 μg/ml) at room temperature for 2hr. in Na^{+}-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (in mM: 140 NaCl, 5 KCl, 2 $CaCl_2$,1 $MgCl_2$, 10 glucose and 10 HEPES, final pH 7.2).

Procedures for Laser Scanning Confocal Microscopy

After washing, one section was mounted on the glass bottom plate of a home-made stainless steel holder with 0.5ml Na^{+}-Hepes buffer for further studies. Resting, proliferative or hypertrophic zone in the growth plate section was selected under the

transmitted light microscope for confocal microscopy. The change of the fluorescence intensity was measured with confocal imaging system at room temperature. At various time intervals, x-y images of 256 x 256 pixel were acquired with 0.2 μm pixel size at 2.5sec intervals on a Multiprobe 2001 confocal laser scanning system (Molecular Dynamics) which is fitted with an Argon laser (8mW) and a Nikon diaphot inverted microscope. After a stable baseline measurement was obtained, calcium releasing modulators, such as TGF-α (final concentration (FC): 0.1 μg/ml, Amersham, USA), TGF-β_1 (FC: 1.5 μg/ml, Sigma, USA) or parathyroid hormone (FC: 20μM, Sigma, USA) was added into the buffer. A series of scans (2.5 sec/scan) were taken at 2.5-5 sec intervals. In some cases, Ca^{2+} free buffer was used to measure whether the change in intracellular Ca^{2+} level induced by the modulators was due to extracellular Ca^{2+} influx. For testing the stability of intracellular Ca^{2+} in chondrocytes of various zones, serial scanning at 100 sec interval, instead of 5 sec interval were performed.

For the fluorescence determination, an excitation filter with 488 nm wavelength and a long pass emission filter of 510 nm were used. Cells were scanned using a 60x oil objective (1.4 NA, Nikon) with low fluorescence immersion oil ($\lambda 23^{\circ}C$ = 1.515, Stephens Scientifics). The voltage for the photomultiplier tube (PMT) was set at 550-750mV and the diameter of the pinhole before the PMT was 50μm.

Image Analysis

Images were processed and the averaged fluorescent intensity in the subcellular compartment was calculated by an image analysis software (Imagespace 3.03, Molecular Dynamics). For the pseudocolour images, black-blue represents a low fluorescence while orange-red illustrates a high degree of fluorescence.

4.4 RESULTS

4.4.1 Variations of Calcium Levels in Subcellular Regions

The intracellular free calcium distribution of growth plate chondrocytes among different subcellular regions varied according to the maturational stages. In resting chondrocytes, the free calcium concentrated in the nuclear region, while those in the rest of cytoplasm were relatively low (Figure 4.3). For the proliferative chondrocytes adjacent to the resting zone, the intracellular free calcium distribution pattern is similar to the resting chondrocytes, although the cell shape of proliferative chondrocyte was spindle instead of spherical in the resting chondrocytes (Figure 4.4). However, in the proliferative chondrocyte of the maturation zone (adjacent to hypertrophic zone), the free calcium concentrated in the sub-plasma membrane region rather than in the nuclear region. For hypertrophic chondrocytes, the calcium also concentrated in the sub-plasma membrane region (Figure 4.5). There is a general trend for the calcium focused in the nuclear and moved to plasma membrane region during the endochondral ossification.

4.4.2 Evidence of Cellular Exfoliation of Calcium Rich Matrix Vesicles

From the investigation of free calcium in extracellular matrix, we have also found that the calcium distribution patterns were changing during the chondrocyte differentiation. In the resting zone, no free calcium could be observed in the pericellular matrix and other cartilage matrix from the confocal image (Figure 4.6). In the proliferative zone, islands of free calcium were found in the horizontal edges of proliferative chondrocyte beside the chondrocyte columns (Figure 4.7). In the hypertrophic zone, arcs of free calcium were illustrated surrounding the hypertrophic

chondrocyte with a distance (Figure 4.8). The calcium arc from one hypertrophic chondrocyte was interrupted by the adjacent calcium arcs.

4.4.3 Stability of Intracellular Free Calcium

From serial confocal scanning the stability of intracellular free calcium concentration of chondrocyte in various maturation zones was monitored. The calcium level in most of the resting chondrocytes was stimulated during multiple Laser confocal scanning, while small amount of them show no changes (Figure 4.9). In proliferative zone, clonal responses was observed. For the clone of proliferative chondrocytes in the same cell column, they exhibited similar intracellular free calcium stability. The majority of cell column showed declining response in multiple scanning, while increasing of free calcium was observed in some other cell columns (Figure 4.10). In hypertrophic chondrocytes, the intracellular free calcium level was unstable and irregular. Two adjacent cells always demonstrated different response during the test period of 400 seconds (Figure 4.11).

In addition, spontaneous oscillations in cytosolic Ca^{2+} , as shown in Figure 4.12, in growth plate chondrocytes were detected occasionally. Their occurring frequency (probability) in resting chondrocyte, about 5% was higher than proliferative and hypertrophic chondrocytes, about 1-2 %. Cells with such cytosolic Ca^{2+} oscillations would be excluded for further experiments on the effects of Ca^{2+} modulators.

4.4.4 Effect of TGF-α on Intracellular Calcium Activity in Chondrocytes in Various Regions of Growth Plate

From the results of this study, TGF-α (0.1 μg/ml) stimulated the intracellular free calcium of growth plate chondrocytes in all three zones. For resting chondrocytes, application of TGF-α induced a sudden and dramatic increase in intracellular free

calcium (Figure 4.13). Then the calcium level decreased and stabilized at a level higher than the baseline level within 1 minute. Responses of individual resting chondrocytes seemed to be synchronized.

The intracellular calcium stimulating effect of individual proliferative chondrocyte by TGF-α was not synchronized (Figure 4.14). Some cells responded faster while the others responded later. After the peak, the calcium levels of individual cells returned to their baseline levels before the TGF-α application within a short period.

The response of hypertrophic chondrocytes to TGF-α was similar to resting chondrocytes (Figure 4.15). The individual cell responses were in a synchronized manner. However, the decline of calcium level after the peak was very slow.

In order to confirm whether the increase of cytosolic calcium induced by TGF-α was due to external Ca^{2+} influx. Experiment under Ca^{2+} free condition was performed and the result was shown in Figure 4.16. It demonstrated that the effect of TGF-α on cytosolic Ca^{2+} increase was not affected in the absent of extracellular calcium. That indicated that the binding of TGF-α to its receptor could trigger the opening of Ca^{2+} channels on the intracellular Ca^{2+} store and then increase the cytoplasmic Ca^{2+} concentration.

It should be noted that under the Ca^{2+} free condition, the baseline intracellular Ca^{2+} level was lower than those with external Ca^{2+} present. The frequency for detecting spontaneous Ca^{2+} oscillation occurred in resting, proliferative and hypertrophic chondrocytes under Ca^{2+} free condition, which is about 10%, almost double of those in normal Ca^{2+} condition. In addition, when we compared Figure 4.15 to Figure 4.16, it seems likely that cells in Ca^{2+}-free condition gave a faster transient response to TGF-α while those in normal buffer produced a longer sustained response. This implies that external Ca^{2+} should play a role in the maintaining the internal Ca^{2+}.

4.4.5 Effect of TGF-β_1 on Intracellular Calcium Activity in Chondrocytes in Various Regions of Growth Plate

There was a differential response on intracellular calcium in growth plate chondrocytes among various zones of growth plate to TGF-β_1. In resting chondrocyte, all cells showed a four fold increase in calcium level after application of TGF-β_1 (1.5 μg/ml). The calcium level then returned to baseline within 50 seconds (Figure 4.17).

Proliferative chondrocytes adjacent to resting zone were responsive to TGF-β_1 while those adjacent to hypertrophic zone were not. For the responsive cells, the stimulation of intracellular calcium was much more mild (0.2 to 1 fold) when compared with resting chondrocytes. The calcium level also returned to original level within 50 seconds (Figure 4.18).

No response on intracellular calcium to TGF-β_1 was observed in hypertrophic chondrocytes (Figure 4.19).

To confirm whether the increase of cytosolic calcium induced by TGF-β_1 was due to external Ca^{2+} influx. Experiment under Ca^{2+} free condition was performed and the result was shown in Figure 4.20. It demonstrated that the effect of TGF-β_1 on cytosolic Ca^{2+} increase was not affected by the absence of extracellular calcium. This result may indicated that the binding of TGF-β_1 to its plasma membrane receptors could trigger release of Ca^{2+} from the intracellular Ca^{2+} store. It was also noted that under the Ca^{2+} free condition, the baseline intracellular Ca^{2+} level was lower than those with external Ca^{2+} present.

4.4.6 Effect of Parathyroid Hormone (PTH) on Intracellular Calcium Activity in Chondrocytes in Various Regions of Growth Plate

Unlike the TGF-α and TGF-β_1, PTH (20 μM) did not affect the intracellular calcium level of resting, proliferative and hypertrophic growth plate chondrocytes. The results were clearly illustrated in Figure 4.21, 4.22 and 4.23 respectively.

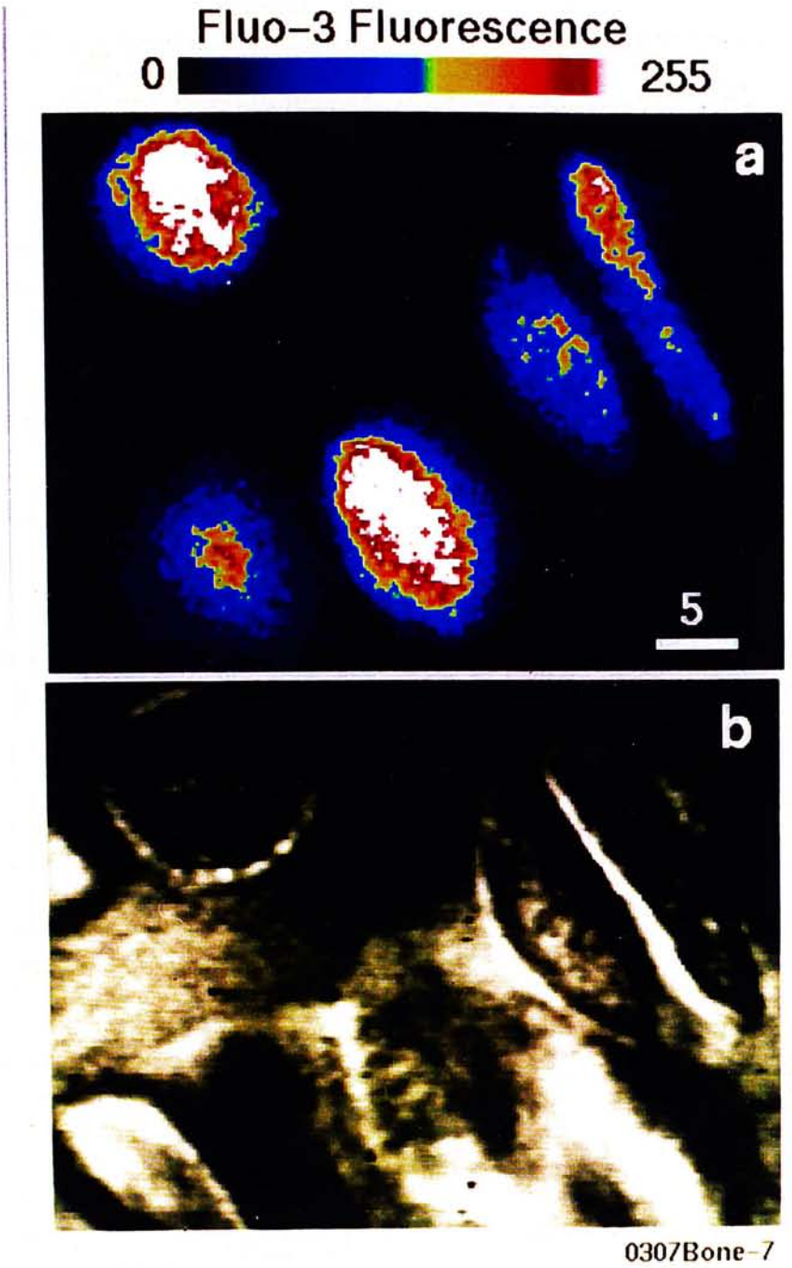

Figure 4.3 Intracellular Ca^{2+} Distribution in Resting Growth Plate Chondrocytes. **a**. Laser scanning confocal fluorescence image: the Ca^{2+} concentrated in the nuclear region, while those in the rest of cytoplasm were relatively low. The colour palette represents the fluorescence intensity. **b.** Transmitted light microscopic image of the same cells in (a.). The white bar represents the cellular dimension in μm.

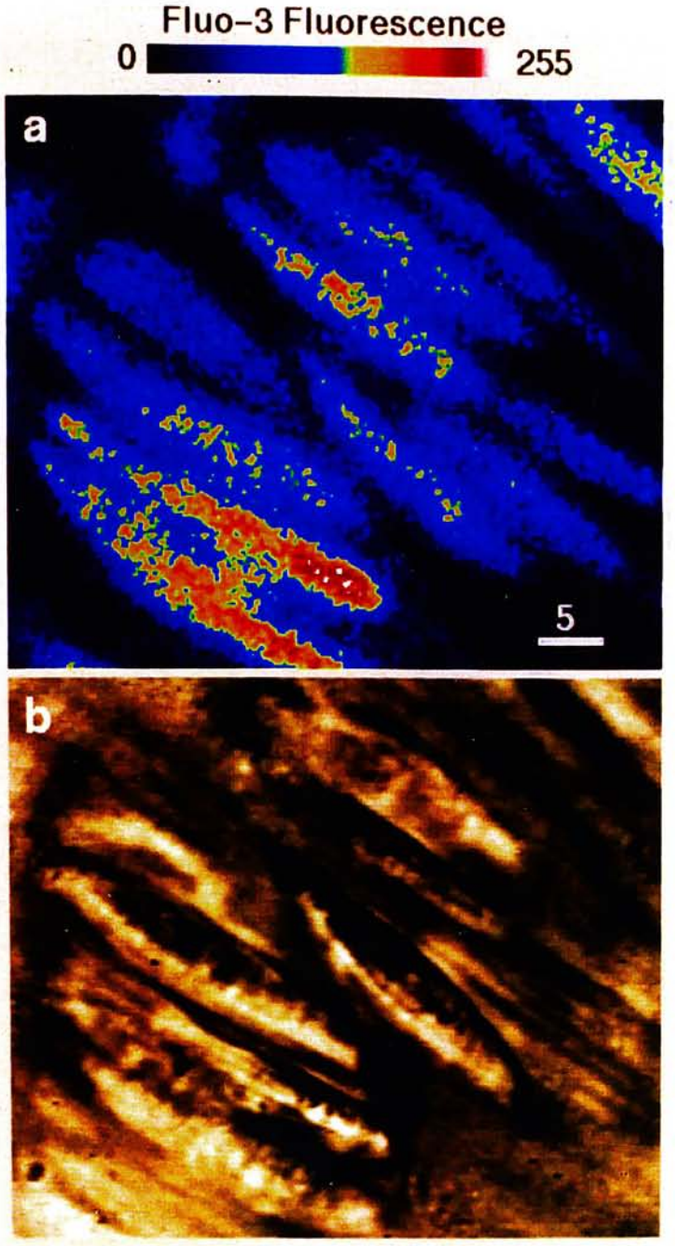

Figure 4.4 Intracellular Ca^{2+} Distribution in Proliferative Growth Plate Chondrocytes. **a.** Laser scanning confocal fluorescence image: For proliferative chondrocytes adjacent to the resting cell, the Ca^{2+} concentrated in the nuclear region, while those in the rest of cytoplasm were relatively low. For those adjacent to hypertrophic zone, the Ca^{2+} concentrated in the sub-plasma membrane region. The colour palette represents the fluorescence intensity **b.** Transmitted light microscopic image of the same cells in (a.). The white bar represents the cellular dimension in μm.

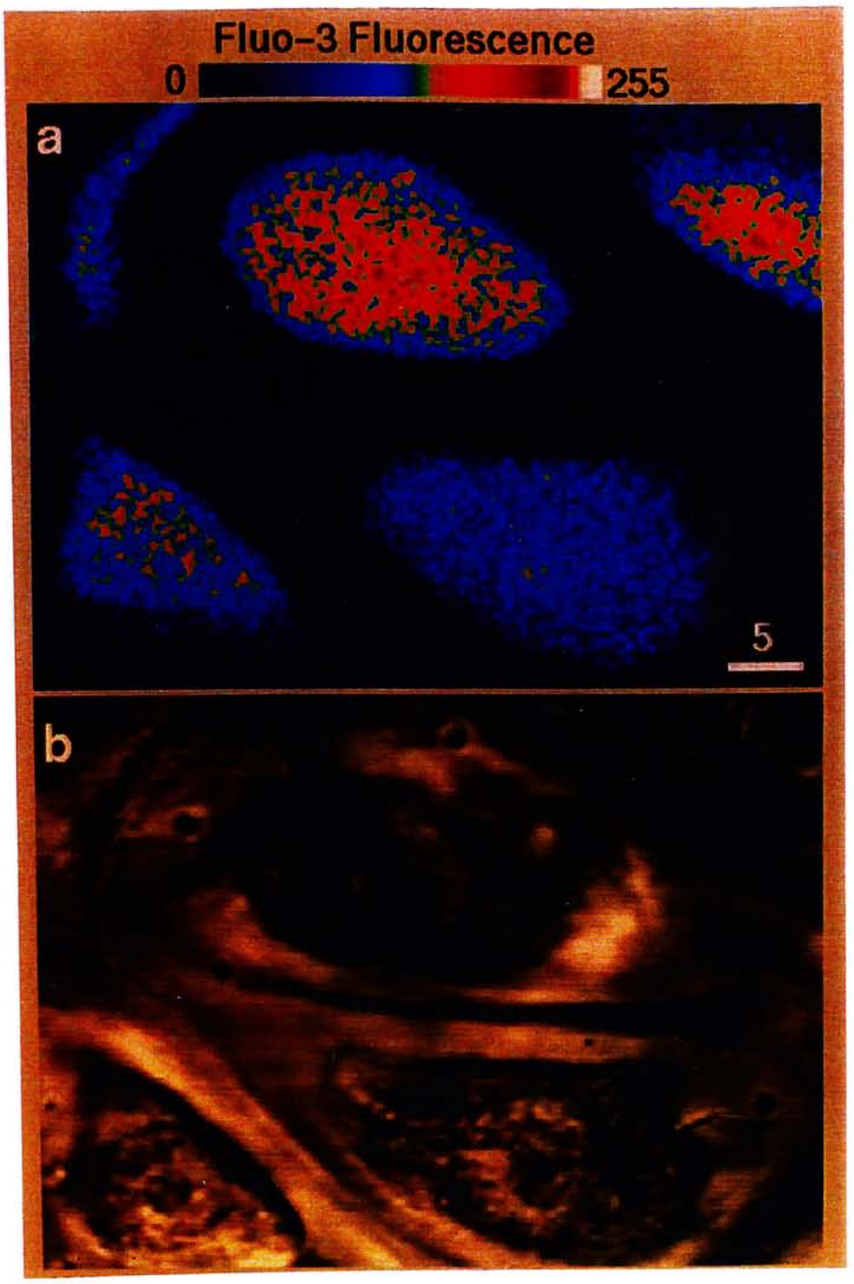

Figure 4.5 Intracellular Ca^{2+} Distribution in Hypertrophic Growth Plate Chondrocytes. **a.** Laser scanning confocal fluorescence image: the Ca^{2+} concentrated in the sub-plasma membrane region. The colour palette represents the fluorescence intensity **b.** Transmitted light microscopic image of the same cells in (a.). The white bar represents the cellular dimension in μm.

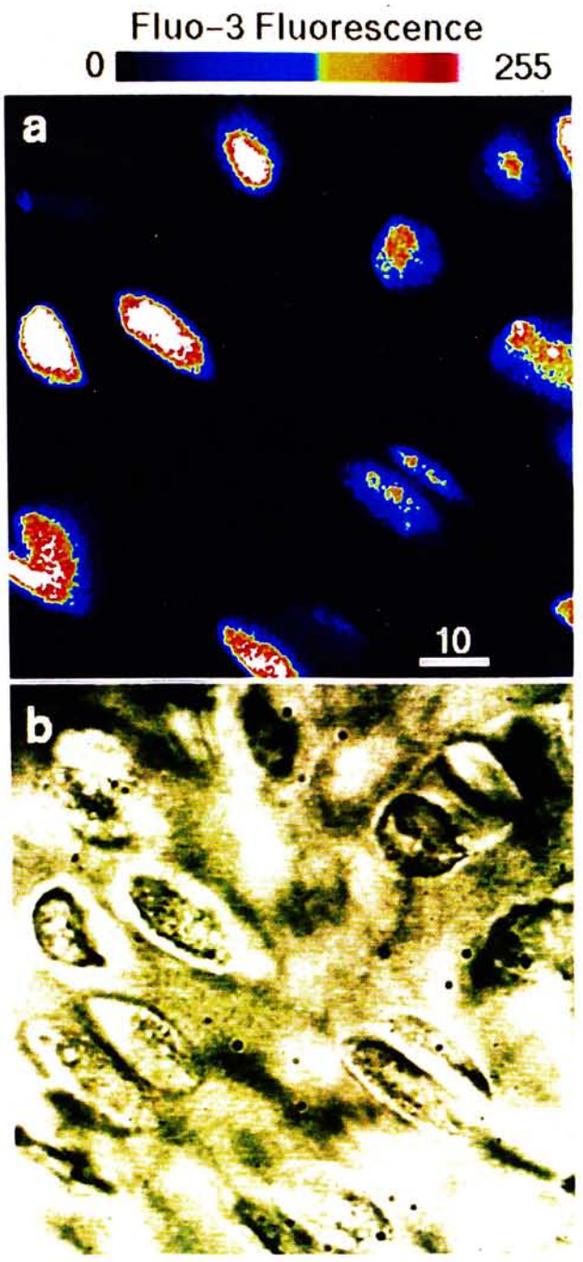

Figure 4.6 Extracellular Matrix Ca^{2+} Distribution in Resting Zone Growth Plate Cartilage. **a**. Laser scanning confocal fluorescence image: the extracellur Ca^{2+} could not be observed in resting cartilage matrix. The colour palette represents the fluorescence intensity **b.** Transmitted light microscopic image of the same cells in (a.). The white bar represents the cellular dimension in μm.

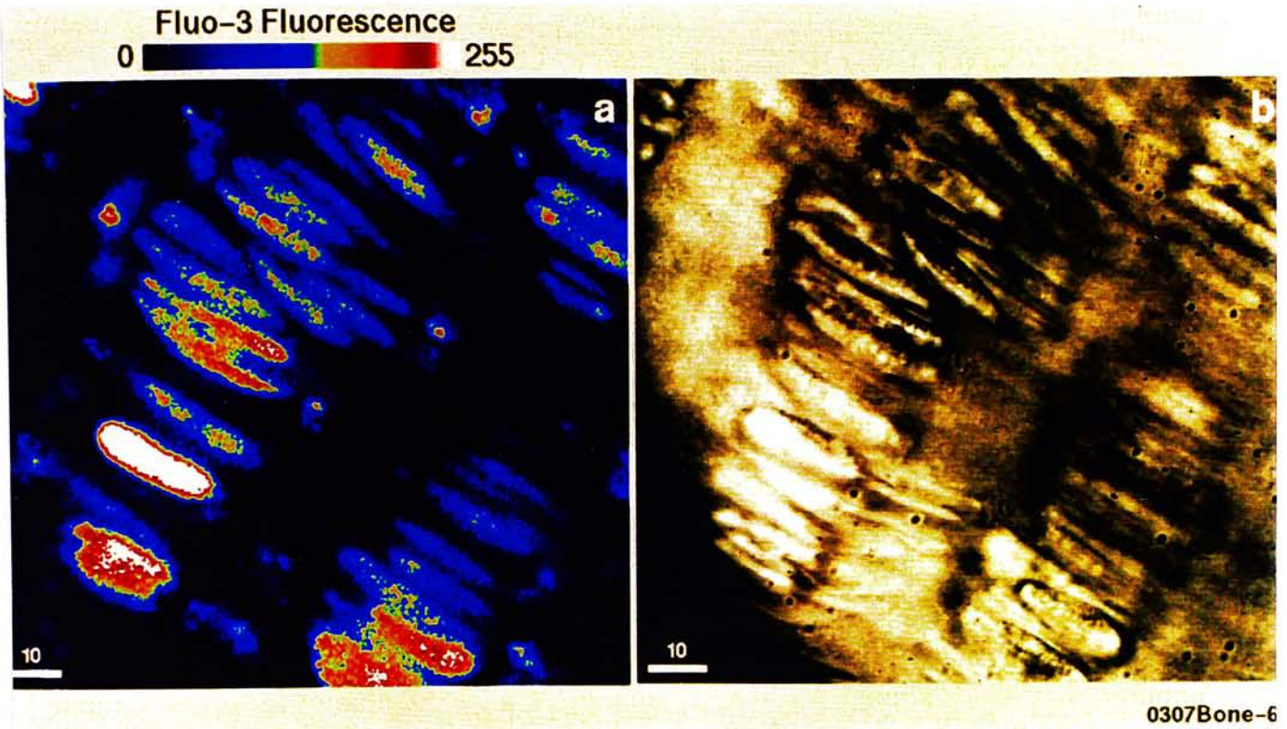

Figure 4.7 Extracellular Matrix Ca^{2+} Distribution in Proligerative Zone Growth Plate Cartilage. **a.** Laser scanning confocal fluorescence image: islands of free calcium were observed in the matrix of horizontal edges of proliferative chondrocyte beside the chondrocyte columns. The colour palette represents the fluorescence intensity **b.** Transmitted light microscopic image of the same cells in (a.). The white bar represents the cellular dimension in μm.

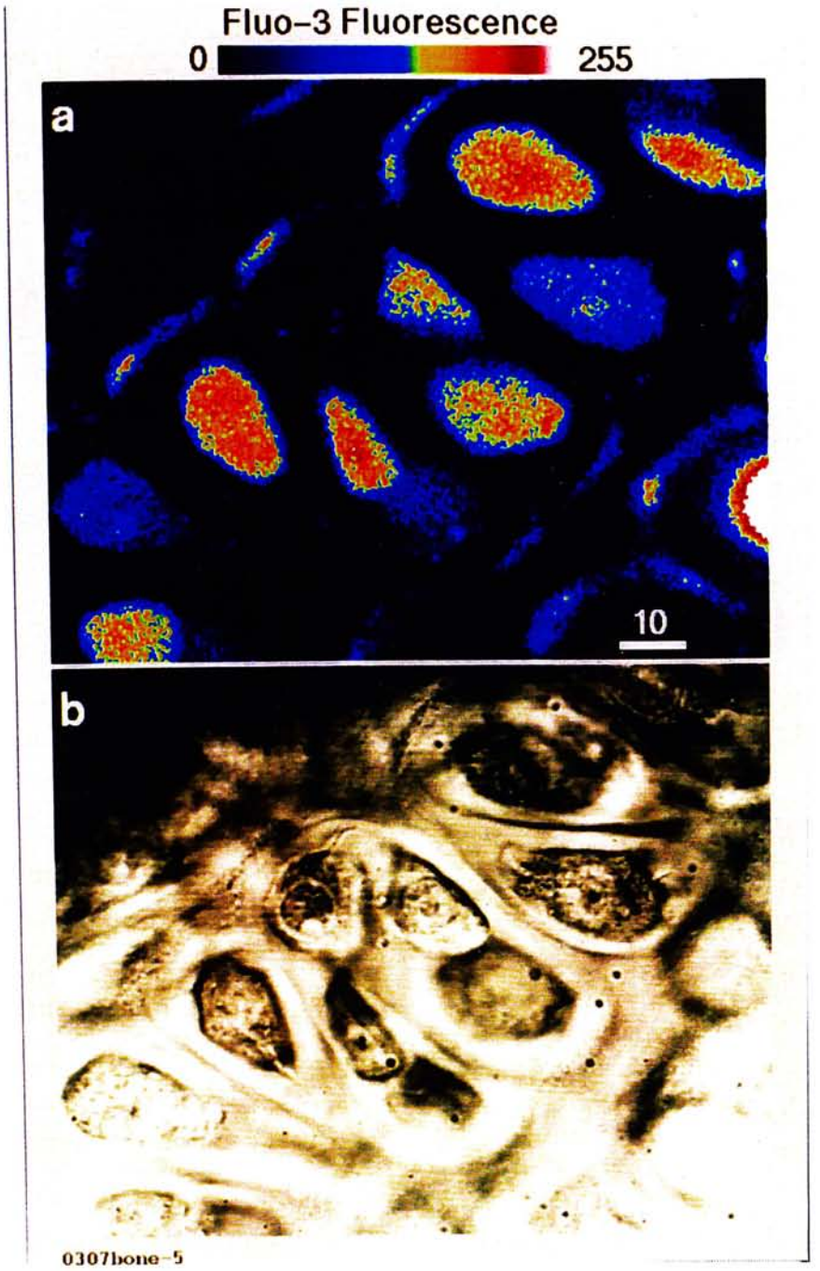

Figure 4.8 Extracellular Matrix Ca^{2+} Distribution in Hypertrophic Zone Growth Plate Cartilage. **a.** Laser scanning confocal fluorescence image: arcs of Ca^{2+} were observed surrounding the hypertrophic chondrocyte with a distance. The colour palette represents the fluorescence intensity **b.** Transmitted light microscopic image of the same cells in (a.). The white bar represents the cellular dimension in μm.

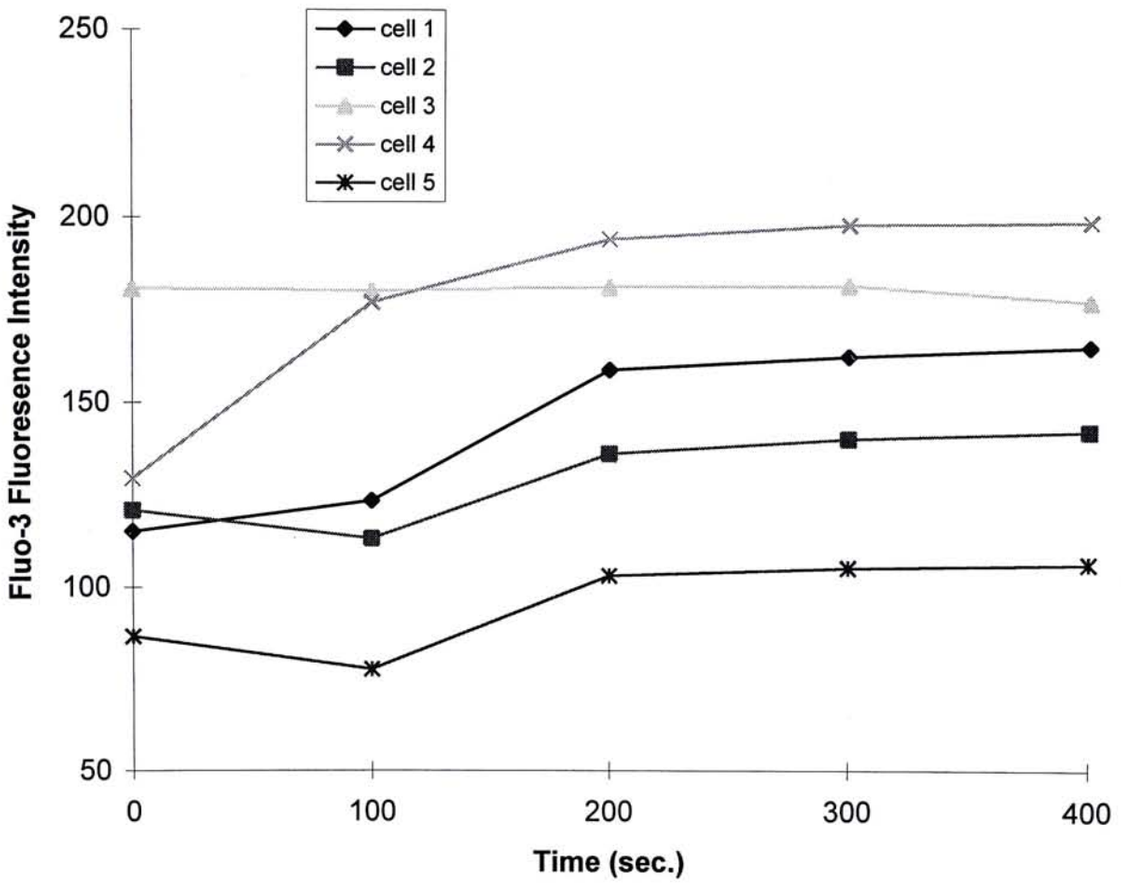

Figure 4.9 Stability of Intracellular free calcium in Resting Chondrocytes. Intracellular free calcium in most resting chondrocytes were slightly stimulated during laser scanning, while small amount of cells showed on changes.
[This experiment is a typical example for 7 trials]

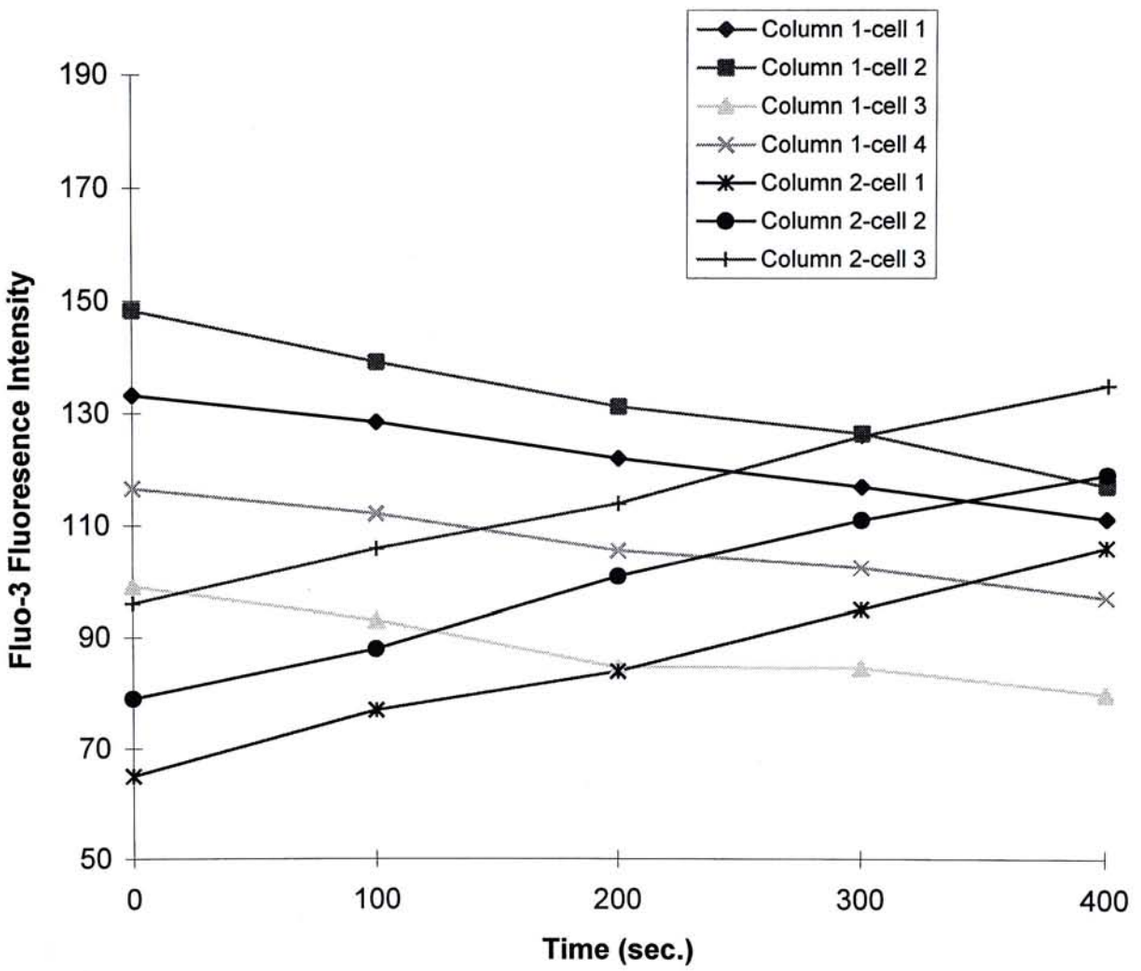

Figure 4.10 Stability of Intracellular Free Calcium in Proliferative Chondrocytes. The majority of cell column showed declining response in multiple scanning, while increasing of free calcium was observed in some other cell columns.
[This experiment is a typical example for 8 trials]

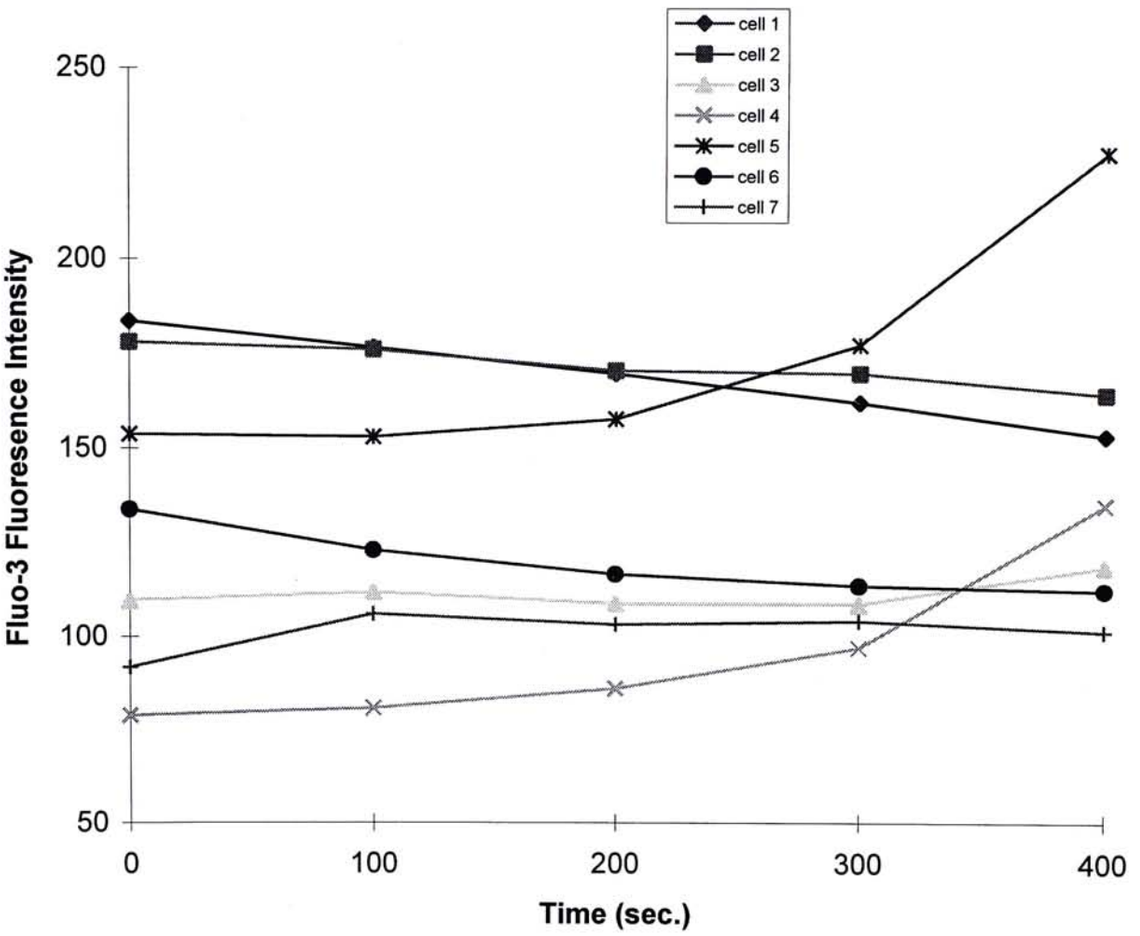

Figure 4.11 Stability of Intracellular Free Calcium in Hypertrophic Chondrocytes. The intracellular free calcium level was unstable and irregular. Two ajdacent cells always demostrated different responses.
[This experiment is a typical example for 7 trials]

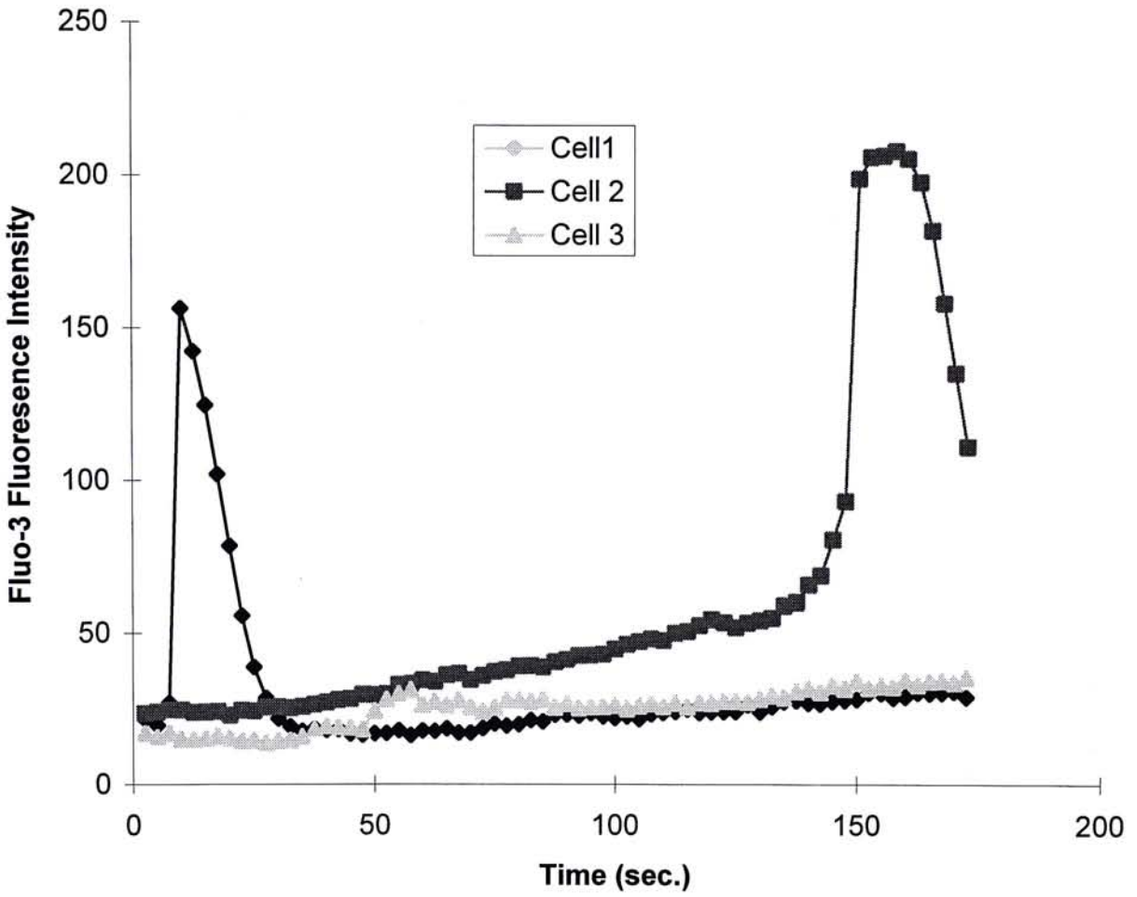

Figure 4.12 Spontaneous Oscillations of Intracellular Free Calcium in Growth Plate Chondrocytes.

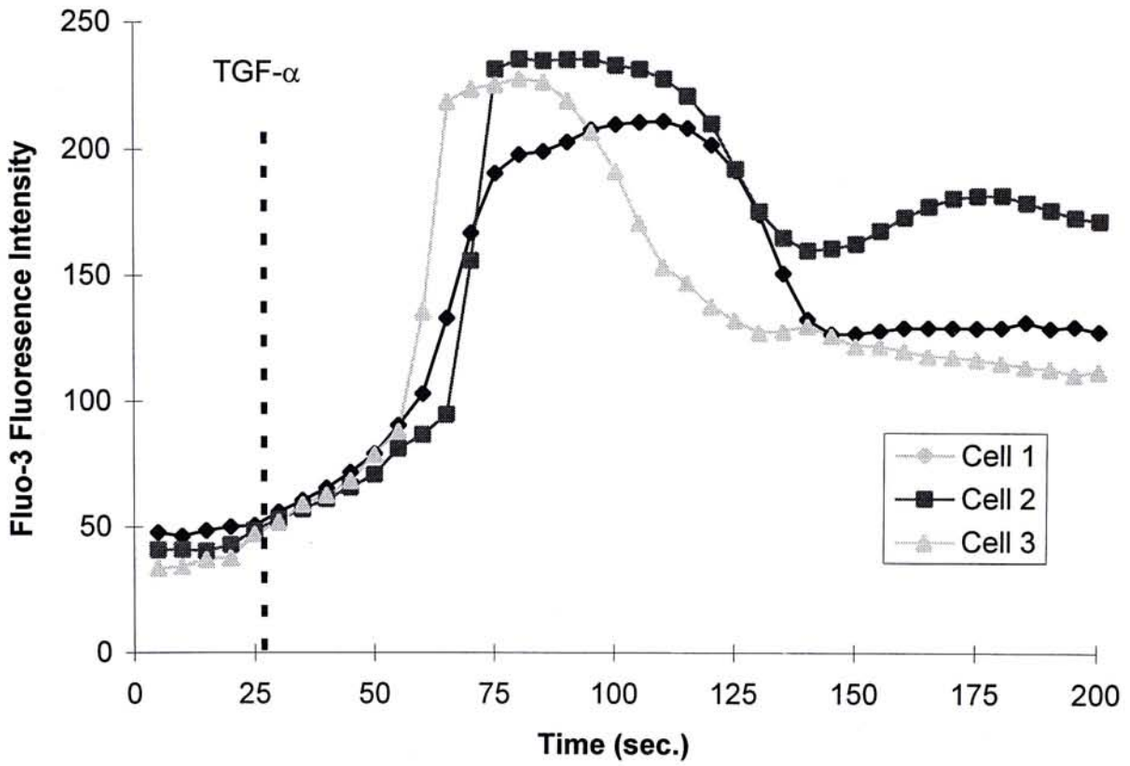

Figure 4.13 Effects of TGF-α on Intracellular Free Calcium of Resting Chondrocytes. Application of TGF-α to a final concentration of 0.1 μg/ml induced a synchronized increase in intracellular free calcium.

[This experiment is a typical example for 8 trials]

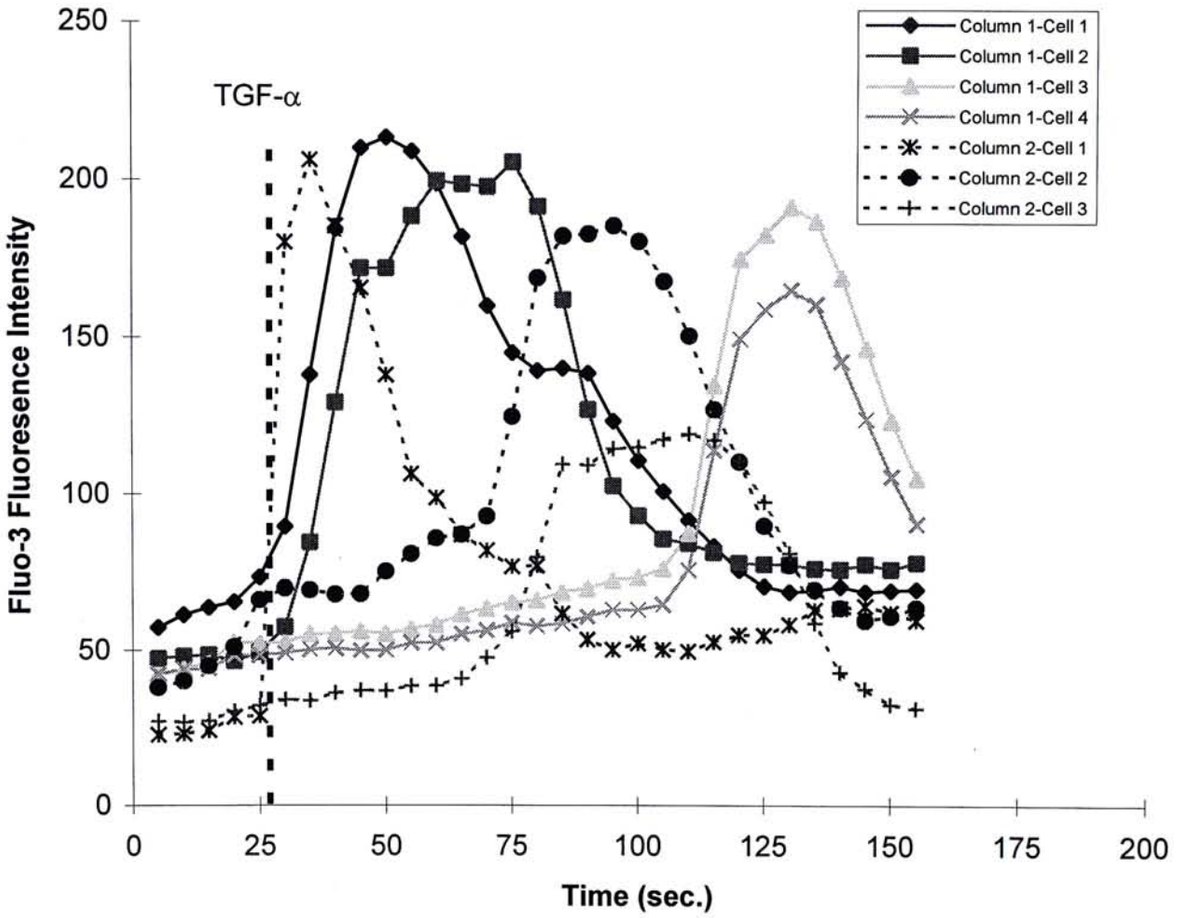

Figure 4.14 Effect of TGF-α on Intracellular Free Calcium of Proliferative Chondrocytes. Application of TGF-α to a final concentration of 0.1 μg/ml induced an asynchronized calcium raise. Calcium levels returned to their baseline within a short peroid.
[This experiment is a typical example for 9 trials]

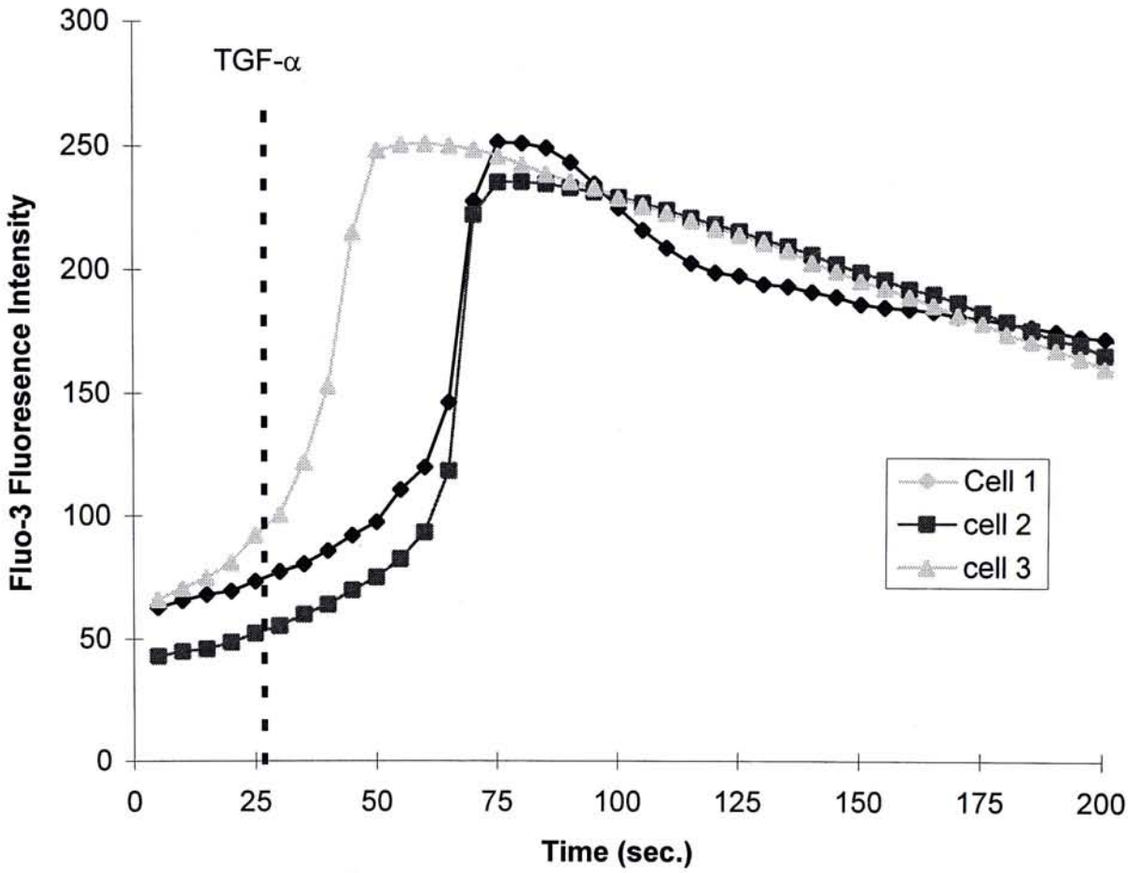

Figure 4.15 Effect of TGF-α on Intracellular Free Calcium of Hypertrophic Chondrocytes. Application of TGF-α to a final concentration of 0.1 μg/ml induced a synchronized increase in intracellular free calcium.

[This experiment is a typical example for 7 trials]

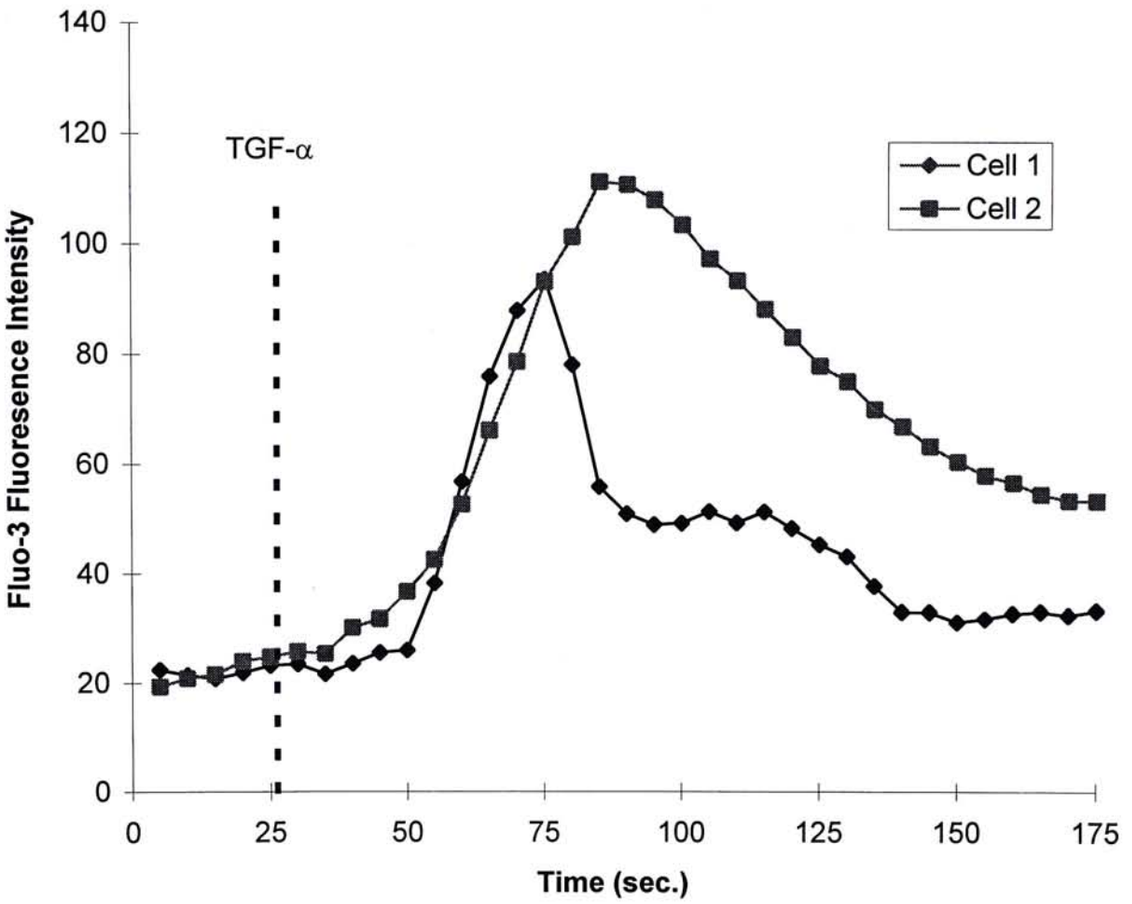

Figure 4.16 Effect of TGF-α on Intracellular Free Calcium of Chondrocytes Under Calcium Free Condition. Application of TGF-α to a final concentration of 0.1 μg/ml in calcium free condition induced a synchronized increase in intracellular free calcium.
[This experiment is a typical example for 8 trials]

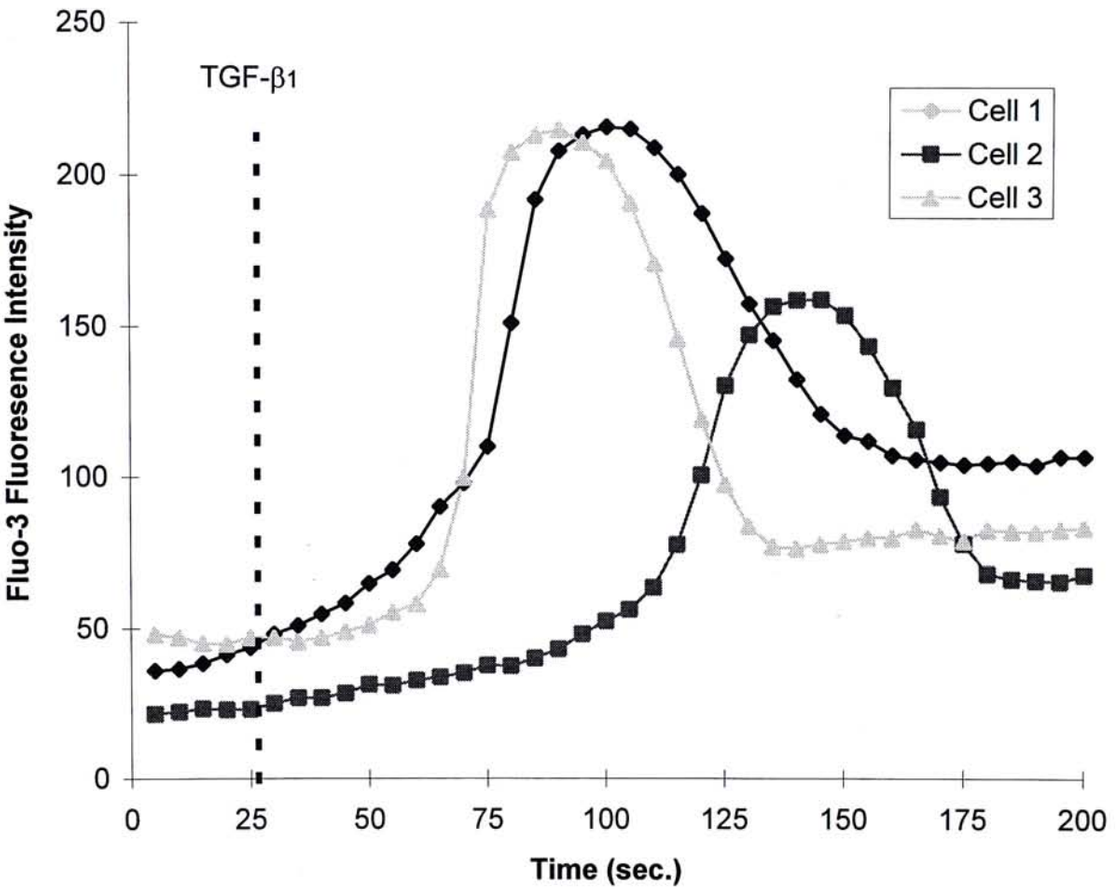

Figure 4.17 Effect of TGF-β1 on Intracellular Free Calcium of Resting Chondrocytes. All cells showed a four fold increase in calcium level after application of TGF-β1 to a final concentration of 1.5 μg/ml.
[This experiment is a typical example for 9 trials]

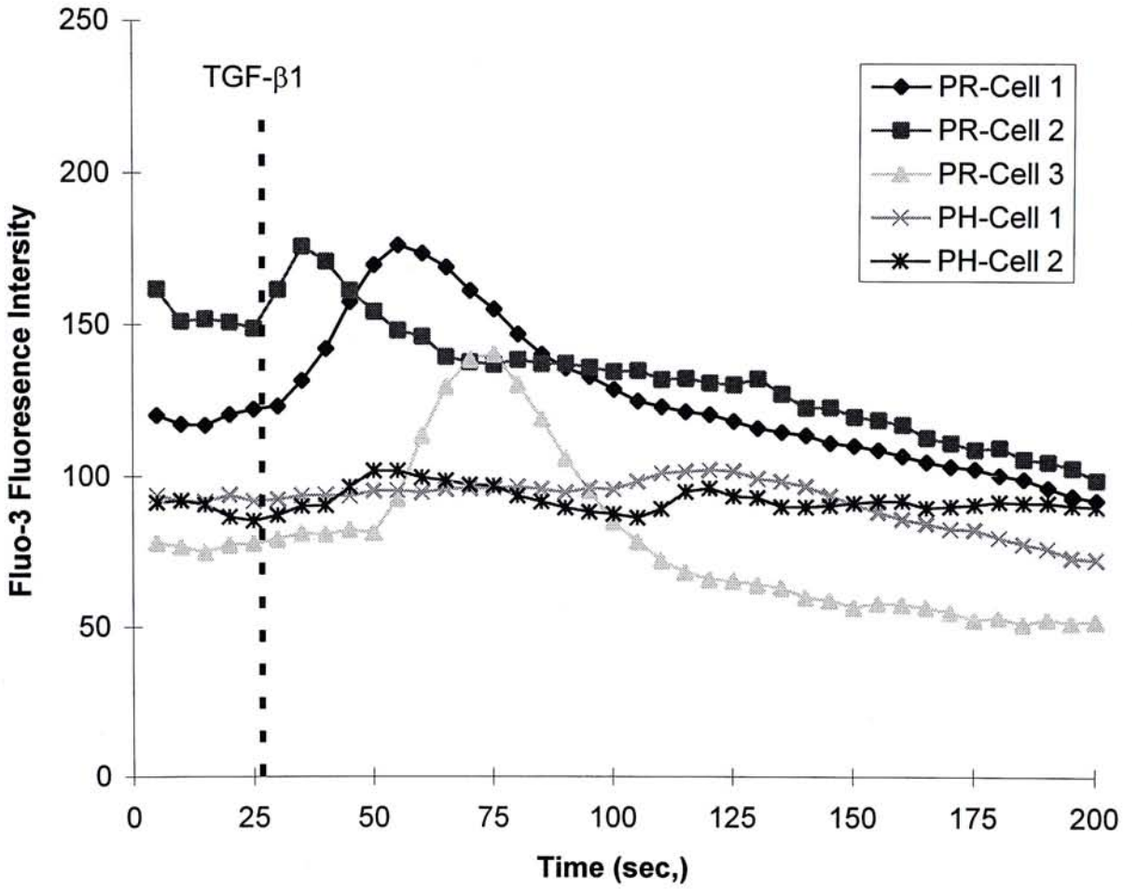

Figure 4.18 Effect of TGF-β1 on Intracellular Free Calcium of Proliferative Chondrocytes. Application of TGF-β1 to a final concentration of 1.5 μg/ml induced calcium raise in proliferative cells adjacent to resting zone (PR) but not those adjacent to hypertrophic zone (PH)

[This experiment is a typical example for 9 trials]

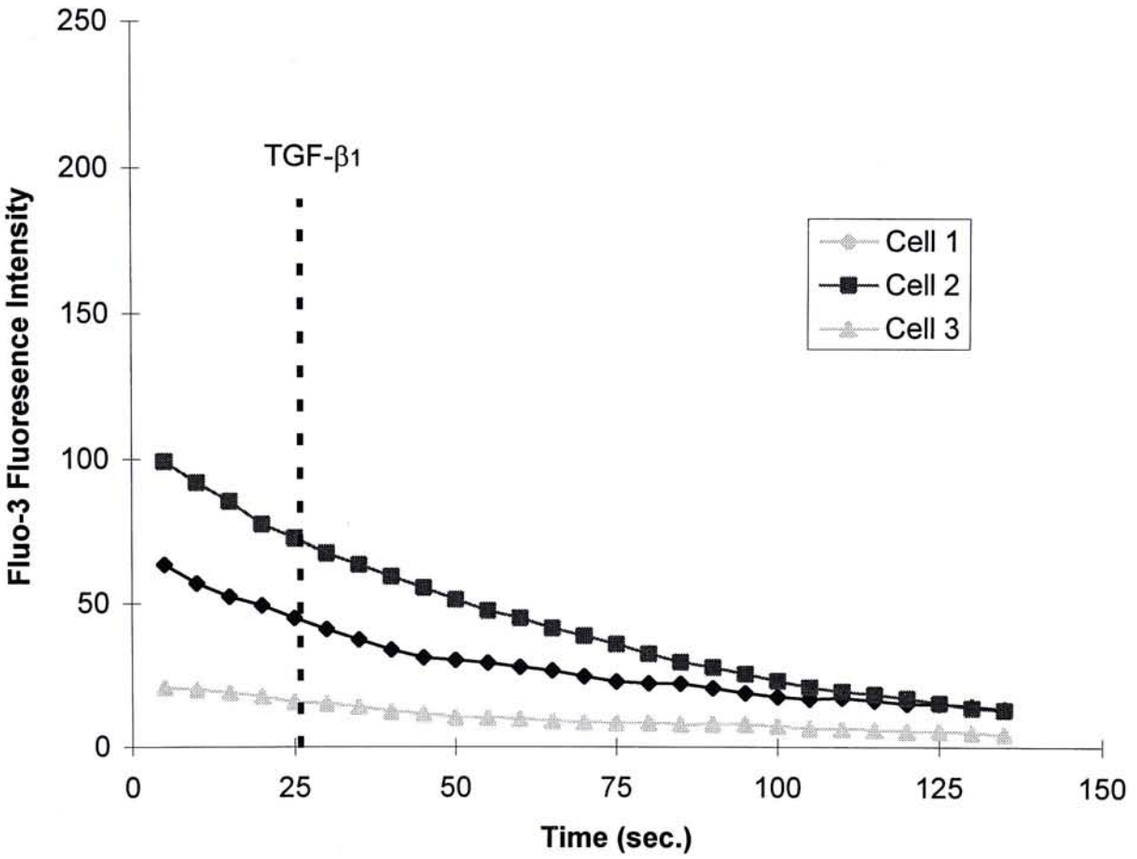

Figure 4.19 Effect of TGF-β1 on Intracellular Free Calcium of Hypertrophic Chondrocytes. No response on intracellular free calcium was observed after application of a TGF-β1 to a final concentration of 1.5 μg/ml.

[This experiment is a typical example for 7 trials]

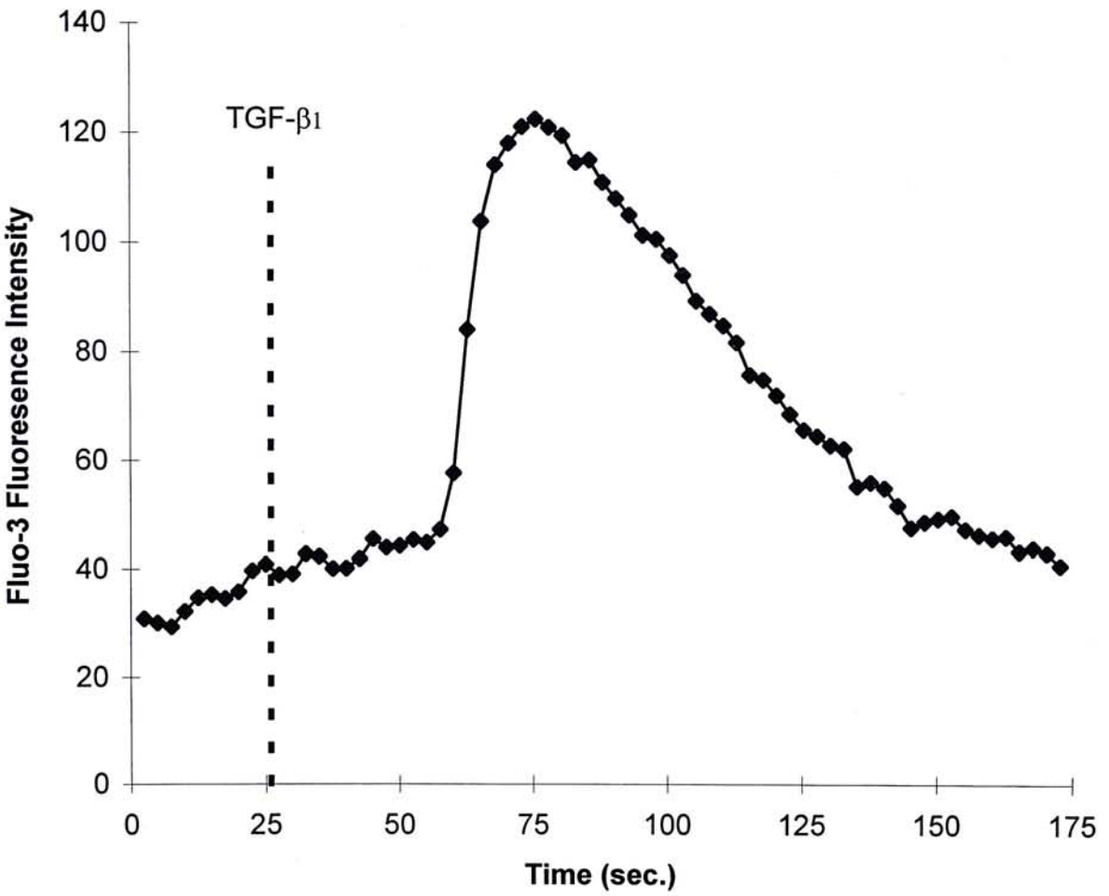

Figure 4.20 Effect of TGF-β1 on Intracellular Free Calcium of Resting Chondroycte Under Calcium Free Condition. Application of TGF-β1 to a final concentration of 1.5 μg/ml induced an increase in intracellular free calcium.
[This experiment is a typical example for 8 trials]

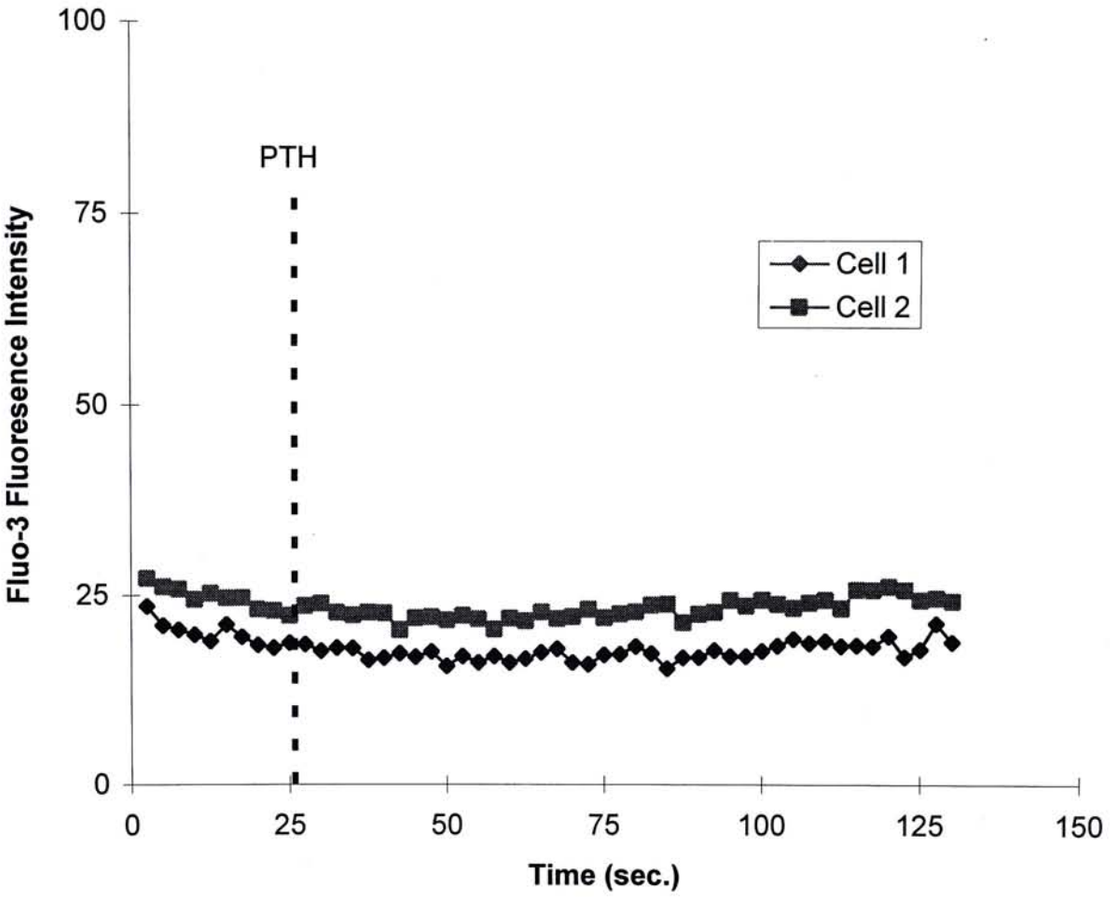

Figure 4.21 Effect of PTH on Intracellular Free Calcium of Resting Chondrocytes. No response on intracellular free calcium was observed after application of PTH to a final concentration of 20μM.
[This experiment is a typical example for 6 trials]

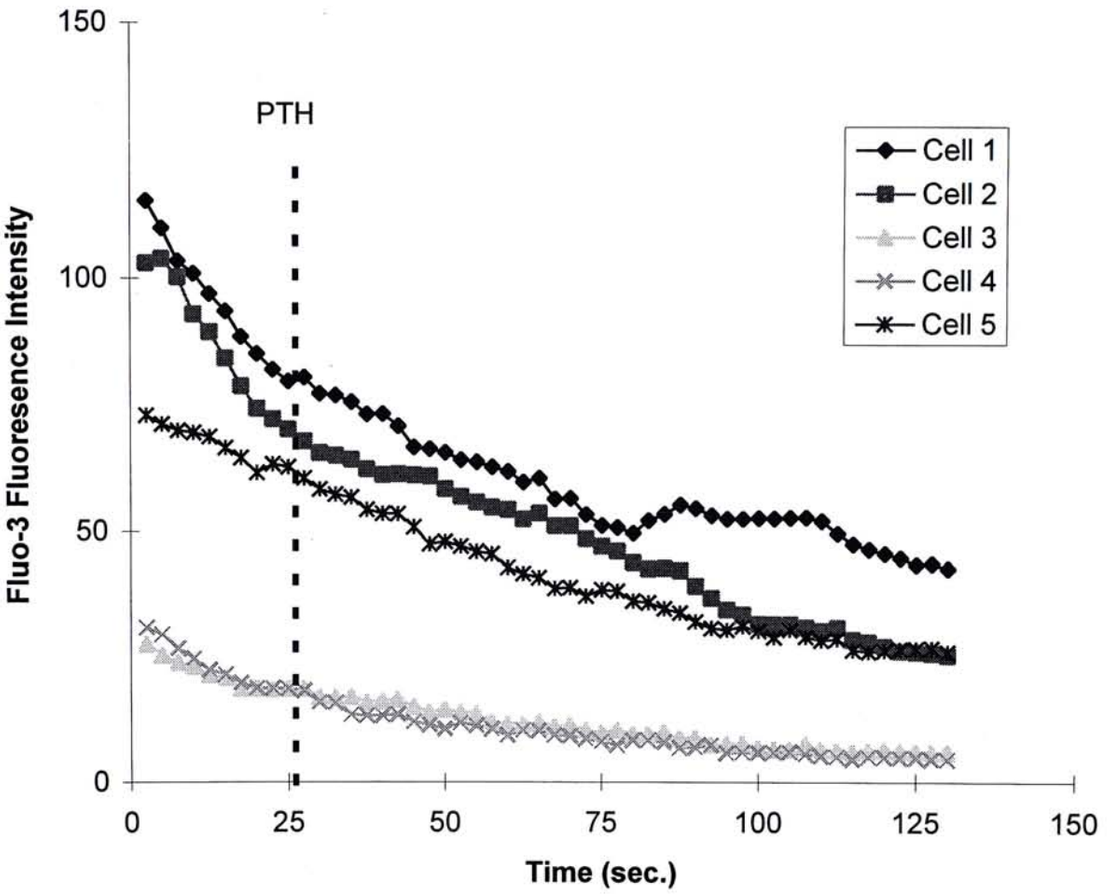

Figure 4.22 Effect of PTH on Intracellular Free Calcium of Proliferative Chondrocytes. No response on intracellular free calcium was observed after application of PTH to a final concentration of 20μM.
[This experiment is a typical example for 6 trials]

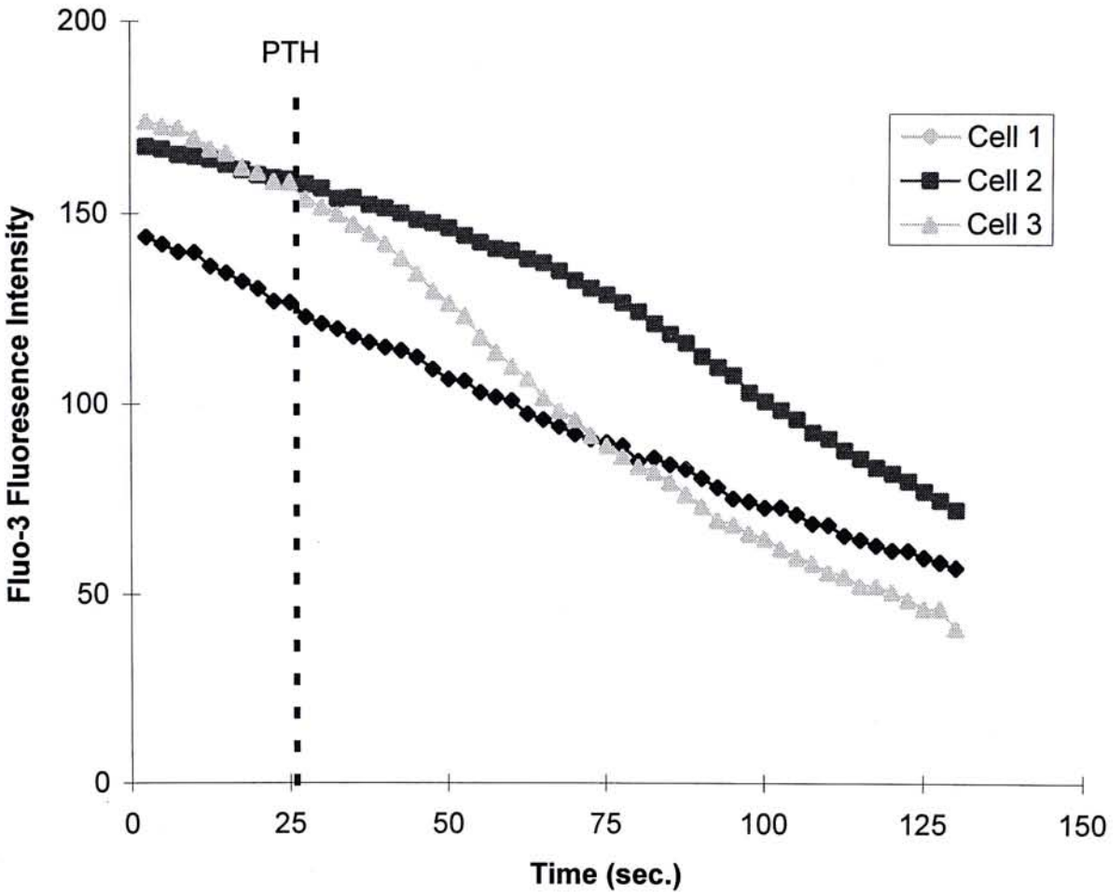

Figure 4.23 Effect of PTH on Intracellular Free Calcium of Hypertrophic Chondrocytes. No response on intracellular free calcium was observed after application of PTH to a final concentration of 20μM.
[This experiment is a typical example for 6 trials]

4.5 DISCUSSION

During endochondral calcification, growth plate chondrocytes undergo a series of biochemical and morphological changes ultimately resulting in calcification of the extracellular matrix. This process, which is the first phase of the transformation of cartilage into bone, is important both in long bone development and in fracture healing.

Calcium metabolism at the cellular level has been implicated in the control of extracellular matrix calcification in cartilage. Electron microscopic studies (Brighton and Hunt, 1976 & 1978) demonstrated a progressive accumulation of intracellular calcium during chondrocyte maturation. They showed that during hypertrophy, calcium localization shifted from the mitochondria to the plasma membrane, accompanied by a parallel increase in the calcium content of extracellular matrix vesicles. Iannotti (1989) has demonstrated increased cytosolic levels of calcium in hypertrophic chondrocytes as compared with chondrocytes from resting hyaline cartilage. The finding of progressive increases in cellular calcium with chondrocyte hypertrophy prior to calcification suggests a potentially important role for intracellular calcium in modulation or initiation of mineralization.

Confocal Imaging of Cellular Calcium Metabolism

Analysis of the progression of biochemical and phenotypic changes occurring during physeal chondrocyte maturation has been limited due to the difficulty of separating cells into distinct populations representing each maturational stage. Recently, Ca^{2+} levels in slices of avian growth plate chondrocytes have been analyzed by laser confocal imaging system (Wu et al., 1992). Cells were maintained in sterile nutrient media and could be examined for extended periods of time (> 1 h) with no apparent

damage as long as laser beam strengths were carefully controlled by proper attenuation and filtering.

From the result of this, there is a general trend for the intracellular free calcium concentrates in the nucleus of the resting chondrocytes through the proliferative chondrocytes to the sub-plasma membrane region in the hypertrophic chondrocytes. It indicates that in the hypertrophic chondrocytes and proliferative chondrocytes of maturation zone, the free calcium is mainly trapped inside the Golgi apparatus and endoplasmic reticulumn for cellular secretion through matrix vesicles. This finding was also supported by the evidence that free calcium arcs and islands were detected in the hypertrophic chondrocyte and proliferative chondrocytes respectively. Moreover, since esterase is required to convert fluo-3/AM to fluo-3, the active form of Ca^{2+} indicator, it suggests that esterase is existed in the matrix vesicles secreted by proliferative and hypertrophic chondrocytes.

Similar result on avian growth plate was reported by Wuthier in 1992. Low power maps of growth plate cartilage revealed a complex pattern, with levels of $[Ca^{2+}]_i$ varying markedly from cell to cell. Although no consistent difference was observed between zones, levels of $[Ca^{2+}]_i$ in cells near penetrating blood vessels were generally elevated. Viewed at higher power, in the zone of proliferation, $[Ca^{2+}]_i$ tended to be lower than in the zones of maturation and hypertrophy, and individual cells had markedly different and variable $[Ca^{2+}]_i$ levels. In the zones of maturation and early hypertrophy, levels of $[Ca^{2+}]_i$ often became intensely elevated in the periphery and in what appeared to be vesicular structures between the cells.

The intracellular free calcium is unstable in various stages of chondrocytes, especially in hypertrophic zone. Calcium level in resting chondrocyte increase in multiple laser confocal scanning. It may indicate that calcium releasing mechanism from the cellular calcium store in resting chondrocyte may be heat sensitive, since it is speculated that laser scanning will generate heat. For the proliferative chondrocyte, we are the first group to report clonal stability of intracellular free calcium. It can be

explained by the fact that one column of proliferative chondrocytes is originated from a single chondrocyte. That is why they show similar calcium activity. The calcium level in hypertrophic chondrocytes fluctuates very much. This may be due to the extremely active calcium metabolism for the matrix mineralization.

These findings are consistent with previous reports. In time studies, overall $[Ca^{2+}]i$ levels in the late proliferative/early maturation zone varied widely within a matter of minutes (Wuthier, 1993). In the maturation and hypertrophic zones, the peripheral Ca^{2+} appeared to be shed from the cells, apparently in the process of forming Ca^{2+}-rich matrix vesicles. These findings extend earlier concepts based on transmission electron microscopy observations of Ca^{2+} distribution in fixed tissues (Arsenault et al., 1988; Brighton and Hunt, 1976; Hunziker et al., 1984), revealing that Ca^{2+} metabolism in the growth plate is remarkably dynamic and indeed may be involved in matrix vesicles formation.

Hypothetical Model of Endochondral Calcification Initiation

Wuthier (1993) has proposed a working model which summarizes the best available information concerning the complex sequence of events in which chondrocytes appear to be directly involved in growth plate calcification.

Growth plate chondrocytes have a very active Ca^{2+} metabolism in which rapid, large overall changes in ionic $[Ca^{2+}]$ in cells, as well as in the subcellular compartments of the cells, occurring within a matter of seconds to minutes.

Growth plate chondrocytes exist in a poorly vascularized, cloistered environment individually surrounded with a dense matrix of proteoglycans and special collagens (Hunziker 1992); this creates a marked diffusion barrier around the cells (Adkisson et al. 1991). The PM of the chondrocytes is attached to the extracellular matrix by

binding of alkaline phosphatase and annexins V and VI to type II and X collagen (Wu et al. 1991 a) and of hyaluronan to proteoglycans (Wu et al. l991b).

Apparently due to the diffusion barrier and avascularity, progressing from the zone of proliferation to the zone of hypertrophy, there is increasing hypoxia (Brighton and Heppenstall, 1971, Shapiro et al., 1982) and ischemia (Adkisson et al., 1991).

Concomitantly, there is a marked increase in the expression of a variety of proteins, most relevant to our discussion here being alkaline phosphatase, type X collagen and annexins II, V and VI. Annexins are cytosolic lipid-dependent Ca^{2+}-binding proteins that form high-conductance Ca^{2+} channels in the PM of the chondrocytes (Rojas et al., 1990; Rojas et al., 1992). Annexins V and VI also bind to type II and X Collagen (Mollenhauer and Von derMark, 1983; Wu et al., l991a) and may be components of stretch-activated Ca^{2+} channels (Wuthier 1992).

Because of the increasing number of Ca^{2+} channels and their attachment to matrix collagens, in the zone of maturation there is entrance of larger amounts of Ca^{2+} into the cells than can be discharged, given the limited available energy resources (Wuthier, 1982). Accumulation of large amounts of Ca^{2+} in their mitochondria (Brighton and Hunt 1976, Matthews and Martin, 1970, Shapiro and Lee, 1975) in the form of amorphous calcium phosphate (ACP) (Posner, 1978) thus ensues.

The combination of localized hypoxia and ischemia and the accumulation of ACP in mitochondria interferes with the synthesis of ATP (Carafoli, 1987; Shapiro and Lee 1975), causing the growth plate chondrocytes to become severely energy discharged, i.e., to have low ATP levels (Hubbard, 1984; Pollesello et al., 1991; Shapiro et al., 1983) and a marked increase in cytoplasmic Pi [20-25 mM] (Wuthier, 1977).

Loss of ATP would lead to failure of the PM Na^{+}/ K^{+} and Ca^{2+}/H^{+} pumps, and the endoplasmic reticulum Ca^{2+}/Mg^{2+}-MPase pumps, allowing further rise in $[Ca^{2+}]$, as well as an increase in cytoplasmic $[Na^{+}]$.

Upon vascular invasion and reperfusion with fresh lymph, the elevated levels of cytoplasmic Na^+ would a) drive additional Ca^{2+} enter through the PM Na^+/Ca^{2+} exchange protein (Carafoli, 1987) and b) cause discharge of mitochondrial Ca^{2+} via the Na^+/ Ca^{2+} exchanger (Carafoli and Crompton, 1978). Both processes would lead to further elevation in cytoplasmic $[Ca^{2+}]$, particularly in the sub-PM region to levels near to those of the extracellular fluid (i.e., 1 mM).

The combination of high cytoplasmic Pi (Pollesello et al., 1991; Wuthier, 1977) and localized high Ca^{2+} (Wu et al., 1992) would cause formation of Ca^{2+}APL-P, (CPLX) complexes (Boskey, 1978; Cotmore et al., 1971; Wuthier and Gore, 1977) associated with the APL-rich inner leaflet (Majeska et al., 1979) of the PM.

Concomitantly, elevated cytoplasmic Ca^{2+} would also cause a) activation of membrane-associated phospholipase A, (Schwartz and Boyan, 1988), forming both lysophospholipids that destabilize the PM and free fatty acids that contribute to the release of mitochondrial Ca^{2+} (Carafoli, 1987); b) attachment (Genge et al., 1989) and insertion (Rojas et al., 1990) of annexin Ca^{2+} channels into the plasma membrane, facilitating further Ca^{2+} influx; and c) depolymerization of actin filaments (Hirokawa et al., 1982) that support cellular microvilli.

These factors, and the aforementioned attachment of the PM annexins to the extracellular matrix collagens, which would stimulate Ca^{2+} influx into the cells during mechanical stress, would contribute to blabbing of CPLX-loaded matrix vesicles (Wuthier and Gore, 1977) from cell membrane microprocesses.

The matrix vesicles so formed would induce calcification of the extracellular matrix (Anderson, 1969; Ali, 1976), and their attachment to the extracellular matrix would facilitate the spread of mineralization into the surrounding matrix. Through this complex series of events, metabolism of Ca^{2+} and Pi by chondrocytes appears to be intimately involved in the initiation of mineralization in growth plate cartilage.

Effect of Transforming Growth Factors on Calcium Metabolism

The transforming growth factors, TGF-α and TGF-β, are two structurally unrelated proteins that were originally copurified in what was believed to be a homogeneous preparation. Subsequently, the biological activity was subfractionated using HPLC into two pools, which turned out to be as distinct functionally as they were structurally.

Transforming growth factor-β_1 (TGF-β) is a polypeptide of 25K originally isolated from transformed cells and described for its ability to confer anchorage independent growth upon normal fibroblasts (Roberts et al., 1980; Anzano 1983). TGF-β has subsequently been found in normal tissues (Roberts et al., 1981), where it has a variety of local regulatory actions (Wahl et al., 1987; Sporn et al, 1986). Whereas the effects of TGF-β are multifunctional, this polypeptide generally functions to stabilize the differentiated phenotype and inhibit cell proliferation (Sporn et al., 1986; Roberts et al., 1986; Knabbe et al., 1987; Takegara, 1987). TGF-β is homologous with a growth inhibitor isolated from kidney endothelial cell culture medium (Holley et al., 1980; Tucker et al., 1984), but in mesenchymal cells acts as a bifunctional regulator of cell growth and may stimulate DNA synthesis under some conditions (Centrella et al., 1987; Fine and Goldstein, 1987; Brown and Holley, 1987). Moreover, TGF-β always stimulates the synthesis of collagen and the deposition of extracellular matrix (Ignotz et al., 1986 & 1987).

TGF-β is found in particularly high concentrations in platelets (Assoian et al. , 1983), activated lymphocytes (Roberts et al., 1986; Kehrl et al., 1986), and bone (Centrella and Canalis, 1985; Seyedin et al., 1985; Centrella et al., 1987). In bone cells, TGF-β is an important regulator of cell metabolism and growth, stimulating collagen synthesis in bone cell cultures and inhibiting or stimulating proliferation depending upon the cell culture conditions (Centrella et al., 1986; Pfeilschifter et al., 1987a). TGF-β is present within bone matrix in an inactive bound form, although

activation may occur under the conditions present during bone resorption (Pfeilschifter et al., 1987b). In bone as well as in other cell systems studied, TGF-β effects are modulated by the presence of other growth factors and hormones (Brinckerhoff et al., 1983; Rizzino et al., 1986; Massague et al., 1985; Gospodarowicz et al., 1987).

The effects of TGF-β have not been extensively studied in chondrocytes. TGF-β is homologous with cartilage inducing factor-A, a protein isolated from bovine bone which stimulates primitive mesenchymal cells to differentiate into chondrocytes (Seyedin et al., 1986). Chondrocytes possess TGF-β mRNA (Robey et al., 1987), and recent histochemical evidence has demonstrated the presence of TGF-β in the developing cartilaginous anlage and its continued presence within calcifying cartilage. TGF-β has also been demonstrated in articular cartilage with immunohistochemical techniques (Ellingsworth et al., 1986). A recent study indicated significant effects of TGF-β on matrix synthesis by articular chondrocytes in cell culture, suggesting a possible important autocrine regulatory role for this growth factor (Skantze et al., 1985).

The presence of TGF-β in calcifying cartilage suggests that it might have regulatory effects on epiphyseal chondrocytes. During the process of endochondral calcification, chondrocytes undergo a series of maturational changes that culminate in hydroxyapatite crystal deposition in the cartilage matrix (Wuthier, 1982; Boskey, 1981; Poole, 1982). The complex sequence of biochemical and morphological changes associated with chondrocyte proliferation and hypertrophic proceeds with remarkable regularity, suggesting the presence of operant local regulatory mechanisms.

Some studies demonstrate that TGF-β has significant effects on epiphyseal chondrocyte matrix synthesis (O'keefe et al., 1988). Epiphyseal chondrocytes undergo a series of exquisitely regulated maturational changes which may be under the control of TGF-β and other growth factors. In addition to the demonstrated

presence of TGF-β in cartilage, other growth factors have been identified in this tissue, including insulin-like growth factors (or somatomedins) (Nilsson et al., 1986) and cartilage-derived growth factor (Klagsbrun and Smith, 1980; Beckoff and Klagsbrun, 1982; Kato and Gospodarowicz, 1985). These factors exhibit regulatory effects on isolated chondrocytes, but to date they have been examined primarily in articular and costal chondrocytes (Kemp et al., 1984; Asakawa et al., 1984; Russell and Spencer, 1985).

The effects of TGF-β on growth plate chondrocytes in various stages of maturation have been investigated with the *in situ* model established in this study. Since most the previous *in vitro* studies on growth plate chondrocyte cell cultures which is actually a mixture of chondrocytes at different maturation stages, the differential responses of individual chondrocytes subpopulations have not been reported. The result of this study demonstrated that there is a differential response on intracellular calcium in growth plate chondrocytes among various zones of growth plate to TGF-β_1. In resting chondrocyte, all cells showed a four fold increase in calcium level after application of TGF-β_1. In proliferative chondrocytes, some cells were responsive to TGF-β_1 while the others were not. For the responsive cells, the stimulation of intracellular calcium was much more mild when compared with resting chondrocytes. There was no response on intracellular calcium to TGF-β_1 in hypertrophic chondrocytes.

Many published data have demonstrated that TGF-β is a potent mitogen for growth plate chondrocytes (Skantze et al., 1985, O'keefe et al., 1988). Therefore, we postulated that the localization of the effectiveness of TGF-β in all the resting and some of the proliferative growth plate chondrocytes may indicate that removal of the TGF-β responsiveness is essential for the triggering of cell division from the resting chondrocytes and further differentiation of the proliferative chondrocytes. Whether it is achieved by disappearance of TGF-β receptor in proliferative and hypertrophic chondrocytes or not required further studies.

By cross-linking radio-iodinated TGF-β molecules bound to the surface of cells, investigators identified three different polypeptides with apparent molecular weights of 55,85, and 280 kDa referred to as types I, II, and III TGF-β receptors, respectively (Lodish et al., 1995). The type I and type II receptors are both transmembrane serine/threonine kinases; binding of TGF-β to a heterodimer containing these subunits is required for signal transduction. Type III receptor is a cell-surface proteoglycan called β-glycan, which appears to regulate the accessibility of TGF-β to the signal-transducing heterodimer of the type I and type II receptor. TGF-β bound to β-glycan serves as an extracellular reservoir of this growth factor and facilitate binding of this growth factor to its cell-surface receptors, thus triggering intracellular signaling pathways. These findings are also consistent with our result that under Ca^{2+} free condition, the effect of TGF-β on intracellular Ca^{2+} in resting growth plate chondrocytes was not affected.

The TGF-α is synthesized by a variety of transformed and normal cells, including retrovirus-transformed fibroblasts (Todaro et al., 1985), cultured cells derived from carcinoma or sarcomas (Derynck et al. 1987), normal skin keratinocytes (Coffey et al. 1987), and normal brain cells (Wilcox and Derynck, 1988). TGF-α is also expressed during fetal development in several tissues. TGF-α is structurally similar to epidermal growth factor (EGF) (about 30% identities in amino acid sequence) and the resulting ability of the two factors to interact with the same receptors (Marquardt et al., 1983, 1984). Actually, TGF-α does not have a separate receptor and mediated its effect through binding with the EGF receptor (Todaro et al., 1980). Whether TGF-α and EGF mediate the same biological effects *in vivo* is at the present time unclear but some reports show they both increase the rate of angiogenesis and wound repair (Scheriber et al. 1986; Schultz et al. 1987), although TGF-α appears to be more potent. The binding of TGF-α to receptor leads to activation of the receptor's tyrosine kinase activity and resulting in increased IP_3 production. Particular attention has been focused on phosphoinositol-bisphosphate (PIP_2)-specific phospholipase C, because its activation has been proposed to trigger two important intracellular

processes: (a) an increase in intracellular Ca^{2+} as a result of release from intracellular stores in response to IP_3 and (b) activation of protein kinase C in response to DAG.

Therefore, triggering of intracellular Ca^{2+} increase by the binding of TGF-α to the growth plate chondrocyte of all the three maturation has been illustrated in this study. The same response occurred under the calcium free condition also confirms that the increased intracellular Ca^{2+} is released from intracellular Ca^{2+} stores.

Moreover, the release of transforming growth factors is a case of autocrine stimulation. TGF-α, for example, is an EGF analog that is released by many tumor cells and binds to EGF receptors, stimulation cell growth. Some TGF-β-superfamily members have anti-proliferative effects on certain tissues and proliferative effects on others.

The literature on demonstrating the physiological effect of TGF-α or EGF on chondrocytes are rare. It has been reported that EGF binding on cultured rabbit chondrocytes stimulates DNA synthesis and inhibits proteoglycan synthesis, suggesting that this factor promotes proliferation at the expense of differentiation (i.e., lineage progression) (Kinoshita et al., 1992). Although the physiological function(s) of the TGF-α binding on growth plate chondrocyte in the present model is still not clear, it is sure that such function(s) are critical and preserved in chondrocyte during their differentiation. This is an important aspect for further investigation.

Effect of Parathyroid Hormone on Calcium Metabolism

Parathyroid hormone (PTH) is secreted from the parathyroid and the thyroid glands. They regulate the plasma calcium level and bone formation. The level of calcium is increased by PTH and stimulates the turnover of bone. In rabbit chondrocyte cultures, this calcium regulating hormone stimulates glycosaminoglycan synthesis, a

differentiated phenotype of chondrocyte (Takigawa et al., 1981). This stimulation is mediated by a rapid and transient increase in the intracellular cyclic adenosine 3',5'-monophosphate (cAMP) (Takano et al., 1987). This rapid and transient accumulation of cAMP is followed by induction of ornithine decarboxylase which is the rate-limiting enzyme in polyamine biosynthesis (Takigawa et al, 1979). The induction of ornithine decarboxylase in response to PTH is also regarded as a good marker of differentiated chondrocytes (Takigawa et al, 1980). However, in the porcine costal growth plate chondrocyte model established in this study, the effect of PTH on chondrocytes does not seem to involve the Ca^{2+} as secondary messenger. Although there is possibility of species specificity of the PTH used may have caused lack of the PTH response in the porcine system. Similar findings that calcium transients are not involved in the transduction of PTH effects on avian growth plate chondrocytes, has been reported recently (Zuscick et al., 1995). Further studies are required to elucidate the mechanism of such PTH unresponsiveness because PTHrP, which is probably the major stimulator of growth plate chondrocytes *in vivo* (Amizuka et al., 1996; Vortamp et al., 1996) and the PTH / PTHrP receptor have been shown in transgenic mice (Amizuka et al., 1994), as well as least one known human growth plate dysplasia, to be absolutely critical for normal growth plate function and maturation (loveys, 1994).

Chapter Five

A New *In Situ* Model
for
Electrophysiological Characterization
of
Ionic Channels
in
Growth Plate Chondrocytes

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5.1 AIMS OF STUDY

Very little is known on the electrophysiological properties of chondrocytes. Using conventional intracellular techniques, it was shown that during differentiation, the resting membrane potential in chondrocytes of the rabbit epiphyseal plate changes along with the potassium intracellular activity (Edelman et al., 1988). Ionic currents could be very important in the maturation of the chondrocytes and other cells. However, no further information is available on the ionic mechanism involved in the regulation of the membrane properties of chondrocytes until Grandolfo and his co-workers first investigated the potassium channels using the patch clamp technique in 1990.

Up to now, all the studies on K^+ channel of chondrocytes were in cell culture model. It was demonstrated that culture time and/or conditions may modify K^+ channels or induce the expression of a new type of channels (Grandolfo et al., 1990). It is necessary to establish an *in situ* growth plate model for further characterization of the existence and properties of various types of potassium channel in chondrocytes. Basically, growth plate chondrocytes refer to a mixture of chondrocytes in various differentiative (resting, proliferative and hypertrophic) stages. These cells will further differentiate or de-differentiate in a culturing condition. In order to investigate the real characteristics of K^+ channel in growth plate chondrocytes, an *in situ* growth plate model is essential.

In the present study we have successfully developed a new preparation of partially digested growth plate which provides cells of identifiable stages of differentiation and which are suitable for patch-clamp recordings, as evidenced by the dramatic increase in success rate of tight-seal formation from virtually 0 up to 40%.

With such a model, at least two types of K^+-channels are found to exist on the chondrocyte membrane and this is consistent with previous findings (Grandolfo et al., 1990 & 1992). We further find out that during differentiation, more outward K^+-currents are expressed which may play critical roles in maintaining a high concentration of extracellular K^+ in the process of mitogensis, cell volume regulation or secretion of Ca^{2+} rich matrix vesicles.

5.2 LITERATURE REVIEW

Ion channels are integral proteins spanning on cell membranes. These membrane proteins have virtual holes inside (which actually serve as tunnels), occasionally allowing ions to pass through, thereby circumventing the hydrophobic barrier of the lipid bilayer that separates cell interior from extracellular space. The transmission of signals within and between cells is mediated by these ion channels. Within the body, ion channels produce the flickers of electrical activity that stir neurons and muscle cells. In sensory organs the channels translate physical or chemical stimuli into electrical signals for the nervous system. Even cells not connected to the central nervous system, such as those in the blood, immune system, liver and other organs, use ion channels for signaling processes (Rudy, 1988). Although the ion channels of chondrocytes were discovered much later, and they are perhaps less well characterized, it is more and more obvious, using patch clamp recording techniques, that a diversity of channel types can also be found in these cells.

5.2.1 Basic Theory of Patch Clamp Technique

Since the 1950s biologists have been able to study the electric currents arising from these ion fluxes at a macroscopic level. Microelectrodes voltage clamp techniques required that either wires or at least two microelectrodes be inserted into a cell, which is generally possible only with the largest types of animal and plant cells. Mammalian cells for example, typically have diameters of not more than 10 to 30 microns and can barely tolerate impalement with a single standard microelectrode. Before the patch clamp technique was developed, only the large cells, such as giant nerve axon and giant algae, can be studied.

The first observation of current flowing through single-ion channels came from studies of biomolecular lipid membranes in the presence of antibiotics (Hladky and

Haydon, 1970). In intact cells, the problem is to detect single-channel currents in the presence of background electrical noise. Conventional intracellular microelectrode methods for current measurement are associated with a background noise of at least 100 pA, whereas the current flowing when a single channel opens is only a small fraction of this background noise.

Neher and Sakmann (1976) solved this problem by the patch-clamp method, for which they received the Nobel Prize for Physiology or Medicine in 1991. Instead of inserting a microelectrode into a cell they pressed a microelectrode tip onto the surface of a cell, effectively isolating a membrane patch. The intrinsic noise increases with the area of the membrane under study, and when a small area (1 to 10 μm^2) is isolated the extraneous noise levels are so low that picoampere currents flowing through single channels can be measured directly.

The patch clamp technique is fundamentally simple. A thin glass pipette of the proper shape is tightly sealed against a cell membrane, thereby isolating a small patch of the membrane and the ion channels it contains. The patch clamp setup is illustrated in Figure 5.1a. These channels can then be chemically or electrically manipulated and their properties deduced. A researcher can even remove a patch of membrane from a cell or carefully open a window into a living cell to alter its cytoplasmic constituents. In all these various applications, the patch clamp technique makes it possible to probe how ion channels affect membrane voltage and cell processes such as secretion and contraction.

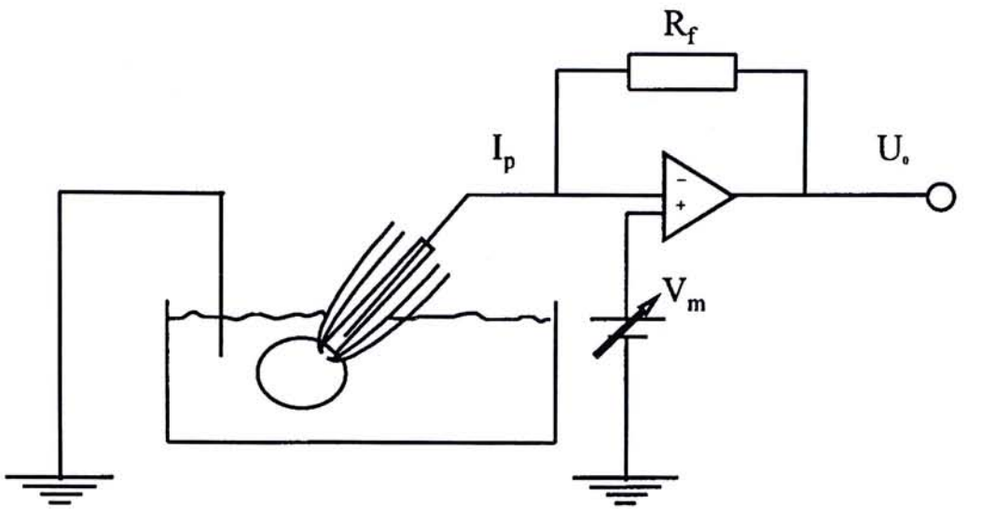
Membrane Configurations in Patch-Clamp Experiments

The seal between the tip of the microelectrode and the outer surface of the cell membrane has, under suitable conditions (fire-polished and clean micropipette tip and clean membrane surface) a high electrical resistance [in the order of giga (10^9) Ohm] and is mechanically very stable. The discovery of this high-resistance seal by

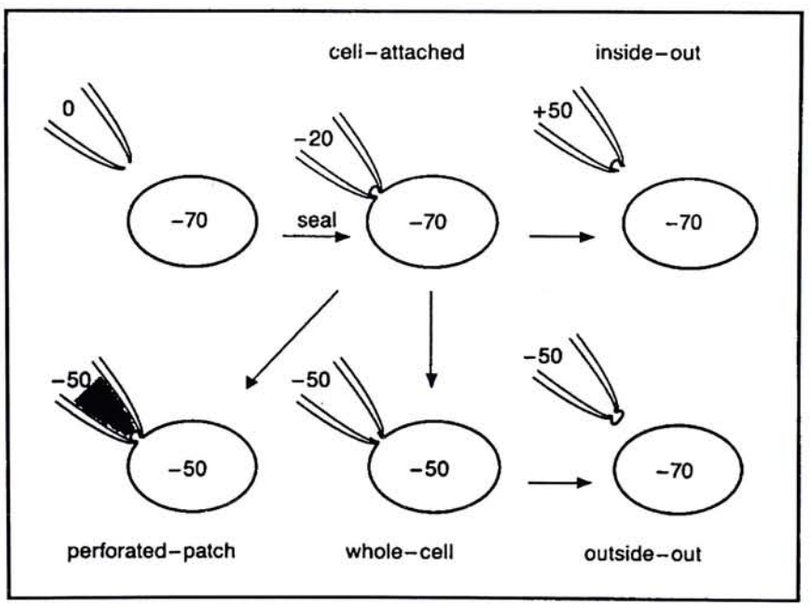
Neher, Sakmann and their co-worker turned out to be very important as it made entirely new types of experiments possible (Hamill et al., 1981).

The electrically isolated membrane patch can be pulled off the cell (excised) in such a way that the inside of the plasma membrane faces the bath solution (inside-out) or alternatively so that the inside faces the solution in the micropipette (outside-out). By breaking the patch membrane in the cell-attached recording conformation, the solution in the pipette interior gains direct access to the cell interior and cell dialysis is carried out under conditions in which the currents across the whole cell membrane can be measured. That configuration, termed whole-cell recording, has been applied in this study. Equilibration of the cell interior with the bath solution can be done while single-channel currents are recorded by making holes in the plasma membrane outside the isolated patch area with the help of detergents such as saponin. The various patch clamp configurations are illustrated in Figure 5.1b.

Whole-cell recording with patch pipettes has become more or less the standard for work with mammalian cell cultures. Even human red blood cells and platelets-cells with only a few microns in dimeter-have been voltage-clamped with patch pipettes. Thus, most cell types of clinical interest have become amenable to a biophysical analysis, and many disorders, such as cystic fibrosis, have been traced back to defects in channel function.



A



B

Figure 5.1 The principles of the patch clamp method. Panel A: The mechanical contact between the membrane and the pipette has high electrical resistance ("giga-seal", 1 - 100 Go). That giga-seal insulates the inside of the pipette from the buffer in which the pipette and the examined cells are immersed. In voltage-clamp mode the membrane potential can be set to any given value (V_m) and the current passing through the patch pipette (I_p) is measured as the voltage drop on a feedback resistor (R_f): U_o-V_m. Panel B: The schematic drawings show the basic configurations of the patch clamp technique. *(Modified from Damjanovich S In: Mobility and Proximity in biological membranes. CRC Press: London, 1994)*

Information Obtained from Patch clamp Recording

In general, ion channels repeatedly open to the same conductance level but that the duration of open and closed periods vary stochastically. Then patch-clamp recording of currents can give very direct information on ion selectivity, control and kinetics of ion channels, and these aspects will be discussed in the following sections.

Ion Selectivity and Channel Classification

Finding out which ions can pass through a particular channel is the first step in the classification process. The ion selectivity can be worked out by measuring single-channel currents over a wide range of membrane potentials using different ion gradients. Further important information is obtained by looking at the shape of the curve relating the amplitude of the single-channel current to the membrane potential.

Control of Channel Opening

Some channels are controlled by the membrane potential, and this class of channel is often referred to as voltage-gated. Such channels may be closed all the time at the resting membrane potential and only be able to make the transition to the open state when the membrane is depolarized. Examples from this group include voltage-gated Na^+, K^+ and Ca^{2+} channels.

Voltage-gated channels can also be controlled by chemical agents. The high-conductance voltage-gated K^+ channel is also regulated by the intracellular ionized calcium concentration ($[Ca^{2+}]_i$). The excised inside-out patch configuration is particularly useful for demonstrating this. In a single membrane patch the influence of varying $[Ca^{2+}]_i$ at different membrane potentials can be investigated, showing that with increasing $[Ca^{2+}]$, less membrane depolarization is required to attain a certain open-state probability (Maruyama et al., 1983). Another channel that can be usefully

studied in the inside-out configuration is the ATP-sensitive K^+ channel (Wang and Giebisch, 1991).

Many channels are controlled by neurotransmitters that interact directly with receptor sites on the outer surface of the channel. The best known example is the acetylcholine-activated cation channel in striated muscle. This was in fact the first ion channel in a native biological membrane that was characterized directly by single-channel current recording (Neher and Sakmann, 1976).

However, there are also channels controlled indirectly by neurotransmitters or hormones acting via second messengers. A clear demonstration of this type of control can be made using the cell-attached recording configuration. Agonist application to the bath solution allows binding to receptor sites on the outer surface of the cell membrane, except the part covered by the patch pipette, and this activates channels to which the agonist has no direct access. With the use of this approach it can be shown that the hormone cholecystokinin (CCK) opens a nonselective cation channel in mouse pancreatic acinar cells via a second messenger (Maruyama and Petersen, 1982). Experiments on excised inside-out membrane patches or open cell-attached patches show that these channels are Ca^{2+}-activated and, as it is known that CCK increases $[Ca^{2+}]_i$; the channel opening would appear to be mediated by Ca^{2+}. This type of experiment has also been used to demonstrate that glucose closes K^+ channels in insulin-secreting cells via a change in intracellular metabolism (Ashcroft et al., 1984).

Kinetics of Channel Opening and Closing

The third type of information that can be obtained from single-channel current recording experiments concerns the kinetics of the opening and closing of ion channels. Dwell times in the open or closed states are stochastic quantities and their means are related to the rate constants of the conformational changes. The simplest possible model deals with only two states

$$C \underset{\beta}{\overset{\alpha}{<=>}} O$$

where C represents the closed state and O the open state; β is the opening and α the closing rate constant. A channel operating according to this model will fluctuate stochastically between the two states. The mean dwell time in state C is equal to $1/\beta$ and the mean dwell time in state O equals $1/\alpha$. Thus by measuring dwell times in the two states, estimates of the two rate constants are obtained (Sakmann and Neher, 1983).

This approach is relatively straightforward when one is dealing with just one channel. However, patch membranes usually contain several non-identical channels. Then the model will be much complicated.

Counting Channels in a Cell

If a cell is totally dominated by one type of channel, one can estimate relatively accurately the number of channels in the cell by comparing results of single-channel current recording with the whole-cell current recording. The whole-cell current I can be accounted for by

$$I = Nip$$

where N is the number of channels per cell, p is the open-state probability and i is the single-channel current; p and i can be determined from single channel current recording, and N is easily calculated from I/ip. In most cases, however, there are several co-existing channel types and it will be necessary to use a pharmacological approach to block, if possible, all channel types except the one under study.

Resting Potential

The Resting Potential is generated mainly by open potassium channels. Cells maintain slightly more negative than positive ions in the cytosol and, conversely, more positive than negative ions in the extracellular fluid. Because of this, a voltage

difference arises owing to the resemblance of the plasma membrane to an electrical capacitor. The potential difference, or voltage gradient, that arises across the membrane thickness (about 0.5 nm) is enormous, nearly 200,000 V/cm. As a consequence, membrane potential and transmembrane ionic gradients provide a driving electrical force for many biologic processes (Figure 5.2).

Membrane potential is based primarily on four ion species: K^+, Na^+, Cl^- and organic anions, such as amino acids and other metabolites. Of these, Na^+ and Cl^- are concentrated in the extracellular fluid, whereas K^+ and A^- are preponderant inside the cell. With the active extrusion of Na^+ from the cell by Na/K-ATPase, the osmotic balance of the cytosol is maintained by preventing the influx of water that would otherwise occur. In exchange for pumping three Na^+ ions out of the cell, two K^+ ions enter the cytosol to counterbalance organic anions that do not permeate the plasma membrane. For a cell with many Na^+ channels in its membrane, such as a nerve or muscle cell, almost all of them remain closed when the cell is at rest, hence, Na^+ extruded by Na/K-ATPase cannot readily reenter the cell down its steep concentration gradient. Only non-gated K^+ leak channels remain open. As a result, K^+ will tend to passively leak from the cell through leak channels down its steep concentration gradient until the force of outward diffusion is counterbalanced by a second and opposing inward electrical force created by the attraction that organic anions in the cytosol have for K^+. Collectively, these two influences-the concentration gradient and voltage gradient for a particular ion across a membrane-determine the net electrochemical gradient that drives the flow of that ion species through a membrane channel. When the electrical and chemical forces counterbalance one another for an ion species, the electrical gradient is zero, and no net flow of this ion occurs across the membrane. Given the concentration of K^+ inside and outside the cell, one can calculate the voltage that is necessary to achieve this equilibrium, termed the equilibrium potential.

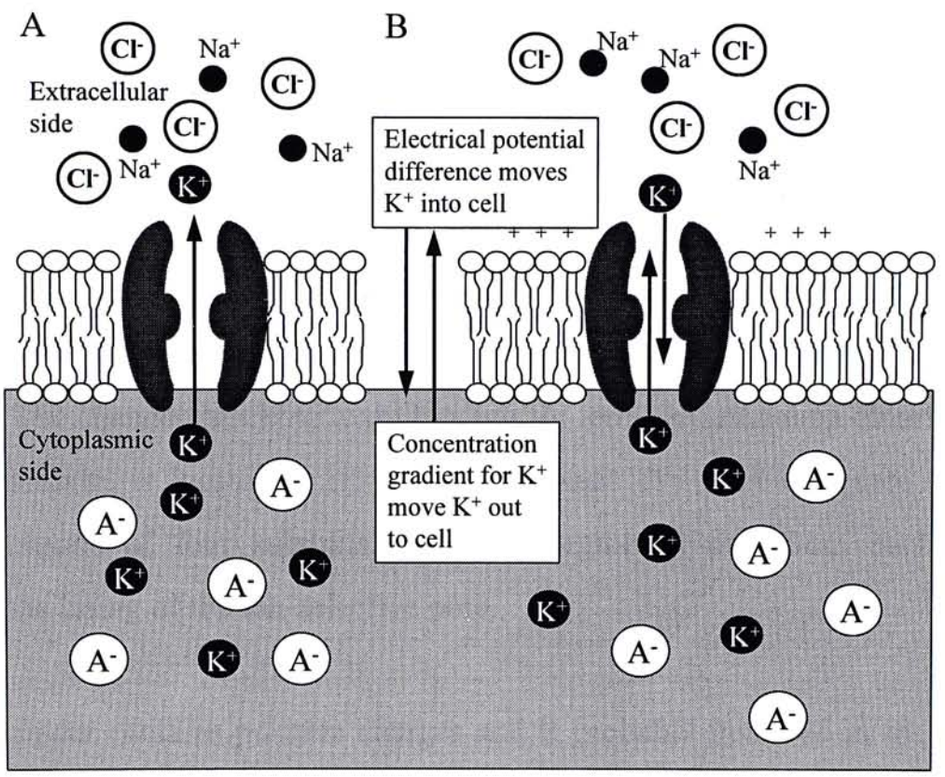


Figure 5.2 Opposing forces regulate K^+ flux across the plasma membrane. **A**. The resting membrane potential of a cell permeable only to K^+ depends on the passive diffusion of K^+ out of the cell down its concentration. **B**. If left unchecked, K^+ efflux would eventually create an excess negative charge in the cell (an overbalance of organic anions) and a build up of $[K^+]_o$ an electrical driving force moving K^+ in the opposite direction. An equilibrium results when these two opposing forces counterbalance each other. *(Modified from Koester J. In: Kandel ER, Schwartz JH, Jessell TM eds. Principles of neural science. 3rd ed. New York: Elsevier, 1991)*

5.2.2 Structure of Potassium Channels

Voltage-gated potassium channels consist of four subunits / polypeptides (Miller, 1991). Each of the four polypeptides that constitute a channel is thought to contain a set of six membrane-spanning α helixes, S1 through S6 and each segment is linked by a hydrophilic sequence (Jan and Jan, 1992) (Figure 5.3). The N-terminus of the polypeptide is in the cytosol, and contains a globular domain, the "ball", that linked to the first membrane-spanning segment by a flexible peptide "chain". The ball is essential for inactivation of the open channel. In addition, there is a hydrophobic sequence between segments S5 and S6 called H5 that is believed to enter the membrane. The pore-lining segment H5 is probably not α-helical. The entire H5 sequence is thought to line the pore itself or its entrance. The H5 segment has been also hypothesized to contribute to the ion selectivity of the channel (Heinemann et al., 1992). The channel-blocking toxin binding for tetraethylammonia chloride (TEA) or charybdotoxin, is located at H5 subunit (Stevens 1991). A complete potassium channel consists of four subunits. Four H5 segments, one from each subunit, constitute the lining of the ion-selective pore.

The S4 segment contains positive charges and is probably involved in the gating of the channel by sensing changes in the electrical field across the membrane (Catterall, 1992). At rest, the interaction of basic (positively charged) amino acids in the S4 helix with fixed negative charges (e.g. organic fatty acids) in the membrane stabilize, the channel in a closed conformation. On depolarization, the introduction of positive charge to the cytoplasmic side of the membrane is thought to repel the positively charged S4 region and cause it to rotate axially and outwardly toward the extracellular surface that trigger the opening of potassium channel (Hall, 1992).

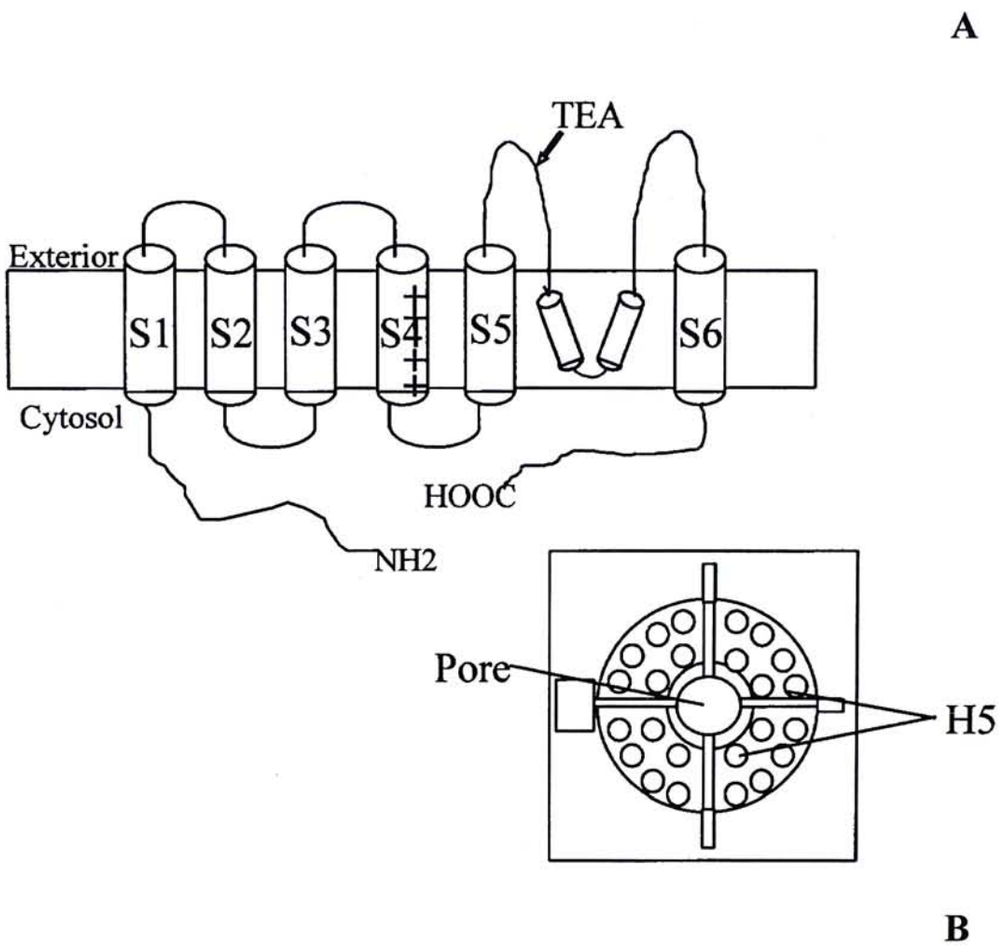


Figure 5.3 **A.** Domain structure and membrane spanning (α-helical segments of the potassium channel polypeptide. Numbered cylinders (Sn) represent the major membrane-spanning segments. S4 contains several positively charged amino acids and is the voltage-sensing α-helix. A possible steric configuration of the four K^+ channel domains forming an active channel in the plane of the plasma membrane is shown in **B**. The TEA inhibitor binding site are also indicated. *(Modified from Damjanovich S In: Mobility and Proximity in biological membranes. CRC Press: London, 1994)*

5.2.3 Types of Potassium Channels

Two classes of membrane transport proteins exemplified for K^+. The influx of K^+ across the plasma membrane is mediated actively by Na/K-ATPase, and passively through K^+ channel-forming proteins.

Active Potassium Channel

In virtually all cells, the concentration of K^+ is higher inside than outside, and the concentration of Na^+ is greater outside than inside the cell. The Na/K-ATPase couples the transport of 3 Na^+ out of the cell and 2 K^+ into the cell. Because these movements are clearly against their concentration gradients, there must be a "driver" to provide energy, and this is achieved by the hydrolysis of one molecule of ATP. The ratio of ions transported leads to an electrogenic potential gradient across the membrane.

Passive Potassium Channel

There is a large spectrum of potassium channel species that have been discovered in the past 60 years. These channels have been identified in many different organisms and tissue types and are involved in various cellular functions. Potassium channels can be divided into four separate categories: voltage-sensitive channels, extracellular ligand-dependent channels, intracellular second messenger-dependent channels and mechano-sensitive channels. The activating stimulus and kinetic properties of the different channel types vary enormously. The involvement of divergent ion channel types allows cells to respond to a variety of stimuli in different ways. Since the voltage-gated potassium channels and Ca^{2+}-dependent K^+ channels are the most important and relevant to this study, each class is briefly discussed below.

Voltage-gated Potassium Channels

Voltage-sensitive ion channels, the most well-recognized class of ion channels, are involved in the voltage-dependent changes in ion permeability; this can result in the generation of action potentials in excitable cells, as well as the control of membrane

potential and ion fluxes in non-excitable cells (Catrerall, 1988). The best characterized of this class of channels are activated by depolarization, and include the well-known calcium, sodium and potassium channels (Hille, 1992). These channels are characterized by high ionic selectivity, single channel conductances (up to 10^7 ions per second), and steep voltage-dependence in channel activity.

An example of the function of these ion channels is the control of the action potential in nerve cells. The activation of the sodium channels increases the Na^+ permeability of the cells, and results in the depolarizing plateau of the action potential. The continued depolarization of the cell activates potassium channels, which result in the efflux of K^+ ions and a repolarization of the cell. This terminates the action potential.

Calcium-activated Potassium Channels

High conductance Ca^{2+}-activated K^+ (PK_{Ca}) channels are found in a wide variety of cell types (Petersen and Maruyama, 1984). There is a high density of these channels in membranes of vascular, uterine, airway, and gastrointestinal smooth muscle and secretory cells (Walsh and Singer, 1987; Barros et al 1991). A primary function of PK_{Ca} channels is to regulate the influx of Ca^{2+} via voltage-dependent Ca^{2+}-channels. Since PK_{Ca} channel activity increases with both membrane depolarization and increasing levels of intracellular calcium ($[Ca_i]$), these channels act as a negative feedback system to counteract depolarizing signals that elicit an influx of Ca^{2+} and an elevation of $[Ca_i]$. Therefore, increasing activity of PK_{Ca} channels will oppose most types of excitatory signals of contraction in smooth muscle and secretion in secretory cells. Drugs designed to interact with PK_{Ca} channels would have potential value for treatment of a number of disorders such as hypertension, preterminal labor, spasms, and asthma. Specifically, for PK_{Ca} channels in smooth muscle, agents should be sought that increase channel activity either by acting directly on the channel or by affecting its regulatory processes. While in secretory cells, agents that inhibit channel activity would be secretogogues.

The Ca^{2+}-dependent K^+ channels are in many different cell types, as illustrated in section 5.2.5; they are also involved in a variety of functions within the cells. It became readily apparent during the identification and characterization of these channels, however, they could be subdivided into several groups based on their conductance, kinetics of opening as well as closing, and the binding of specific channel blockers. Three primary groups of Ca^{2+}-dependent K^+ channels that have been described. The names given to these groups are the Maxi K channels (or sometimes referred to as the BK channels for big conductance), the SK channels (small conductance) and IK channels (intermediate conductance).

5.2.4 The Function of Potassium Channels

In excitable tissues, such as muscle and nerve, K^+ channel currents play an important role in regulating cellular electrical activity by maintaining the cell resting potential and controlling the action potential duration (Hille, 1984, Kass et al, 1990). The existence of K^+ channels in a wide range of non-excitable cells has prompted speculation that these channels may also serve other important functions. In the immune system, K^+ channels may be involved in T lymphocyte activation (Chandy et al., 1984; DeCoursey et al., 1984; Matteson and Deutsch, 1984), lymphocyte volume regulation (Cahalan, 1988), and macrophage phagocytosis (Ypey and Clapham, 1984). In fat cells, K^+ channels may regulate hormone-mediated lipolysis (Lucero and Pappone, 1989). Additionally, K^+ channels in bone and growth plate cells may be required for proper mineral metabolism (Ypey et al., 1988; Ravesloot et al, 1989 & 1990).

The existence of Ca^{2+}-dependent K^+ channels in such a large variety of tissues and organisms suggests both important and multiple functions. Indeed, they are thought to be involved in such actions as the repolarization of action potentials, the spike-frequency adaptation of neuronal cells, the control of secretion, the setting of the

membrane potential and the regulation of arterial tone, among other functions. We will now briefly review the evidence for several of these functions.

Fast and Slow Afterhyperpolarization

Many vertebrate neurons experience a rapid series of action potentials, called spike trains, following a single stimulus. Fast and slow afterhyperpolarizations follow these action potentials, which are due to K^+ effluxes. The fast afterhyperpolarization is produced by a voltage-dependent K^+ current in some neurons, such as the motor neurons in humans (Yarom et al., 1985). On the other hand, in other neuronal types the fast hyperpolarization is the result of a Ca^{2+}-dependent K^+ current such as those in hippocampal pyramidal cells, this fast hyperpolarization is blocked by the scorpion toxin charybdotoxin, which is known to block Ca^{2+}-dependent K^+ (Nicoll, 1988). This rapid hyperpolarization helps to repolarize the membrane after the action potential.

Along with the fast afterhyperpolarization, the slow component plays a critical role in the dampening of the spike train in these neurons. The slow hyperpolarization is due to Ca^{2+}-dependent K^+ channels in all neuronal types tested. This current is inhibited when the intracellular $[Ca^{2+}]$ is lowered (Barrett and Barrett, 1976). The slow component is believed to be involved in slowing of the discharge rate of the neurons following a stimulus (Madison and Nicoll, 1984). This phenomenon, called spike-frequency adaptation, is extremely important throughout the nervous system of most organisms (Hille, 1988). A buildup of the slow hyperpolarization eventually prevents the cell from reaching the firing threshold, and shuts off the spike train.

Secretion

The glands, ducts and vessels of animals are lined with epithelia that move salts and fluid. Many ion channels control this epithelial transport (Gorman and Thomas,

1978) often this involves the adsorption of Na^+ and secretion of Cl^- through Ca^{2+}-dependent K^+ channels.

An example of such a secretory event is the epithelia of the airway in animals. The quality and composition of the respiratory tract fluid are controlled by electrolyte transport through the airway epithelia. There has been a great deal of research on the secretion of Cl^-, which has led to the discovery that a defect in these ion channels is responsible for the genetic disease cystic fibrosis (Schoumacher et al., 1987; Li et al, 1988). In airway epithelia, as well as many other secretory epithelia, a Ca^{2+}-dependent K^+ channel appears to be involved in the recycling of K^+ that enters the cells via a Na^+-K^+ transporter. This channel was first identified in cell-attached and excised patches, which reveals the presence of an inwardly rectifying K^+ channel that shows a dependence on Ca^{2+} for optimal activation (Welsh and McCann, 1985). Secretagogues that activate these channels, such as epinephrine and isoproterenol, appear to increase the intracellular [Ca^{2+}] (McCann and Welsh, 1990). The increased calcium in turn activates the Ca^{2+}-dependent K^+ channels. These channels have low ionic conductance and are sensitive to the channel blocker charybdotoxin. Therefore, this class of Ca^{2+}-dependent K^+ channel, acting in concert with other ion voltage- and Ca^{2+}-dependent ion channels, is involved in the control of Cl^- secretion in epithelial membranes.

Cell Volume Regulation

Lymphocytes regulate their volume in response to anisotonicity. In hypotonic media they swell, and then shrink back toward isotonic volume while still in hypotonic media (Deutsch et al., 1982). This response is known as regulatory volume decrease (RVD), and has been extensively documented by Grinstein and co-workers (1984).

Quinine, a K^+ channel blocker, inhibits this RVD process in a mouse helper T-cell line, L2 and Gramicidin, a monovalent cation ionophore, however, accelerates RVD in the presence of an impermeant extracellular cation (Deutsch, 1990).

Cell growth is a coordinated and integrated process, which includes increases in cell volume and membrane surface area. The surface area increases at least 2-3 fold in stimulated human lymphocytes and mouse cells during mitogenesis. This requires synthesis and insertion of membrane components. New lipid and protein could be incorporated into the plasma current density following mitogen-activation, and agents such as quinine, TEA, and 4-aminopyridine (4AP), block the K^+ channels in the patch-clamp experiments as well as proliferation in culture (Deutsch, 1990).

Mitogenesis

K^+ channels play a role in stimulated proliferation. Potassium channels underlie the signaling mechanisms responsible for routine cellular activities such as cell-cell communication, secretion and conduction. Some of these functions may likewise be involved in cell proliferation and differentiation.

There are several lines of evidence for the voltage-gated K^+ channels of lymphocytes that may play a part in mitogenesis. Firstly, Cahalan and co-workers (DeCoursey et al., 1984) reported a shift in the conductance vs. voltage curve such that more K^+ channels are open at potentials negative to -20mV when the cells were treated with specific lectin mitogens, such as PHA and ConA, all of which stimulate these cells to proliferate. For the longer-term stimulation with mitogens on cell culture, increase in DNA synthesis as well as K^+ current per cell were demonstrated.

Moreover, modulation of potassium conductance is accompanied by altered proliferation. Agents that block the K^+ channel, such as TEA, 4AP, quinine, verapamil and diltiazem, inhibit stimulated DNA synthesis with the same potency sequence, near, but at slightly higher K_i (Chandy et al., 1984; Lee et al., 1986). There appears to be a threshold level of channel blockage: at least 95% of the K^+ channels must be blocked in order to inhibit proliferation. The difficulty with these experiments is that we do not know the *in situ* functional status of the conductance.

We only know the characteristics of these channels during the patch-clamp experiment.

5.2.5 Distribution of Potassium Channels

In the last 15 years there has been an explosion of literature describing Ca^{2+}-dependent K^+ channels from a variety of sources. Surprisingly, these channels were found to exist in nearly every vertebrate cell type examined. Table 5.1 provides a partial list of the Ca^{2+}-dependent K^+ channels that have been described in the literature from various organisms and tissues. From Table 5.1, one gets the vivid impression of a wide distribution of these channels throughout the biological kingdoms; they are seen in a wide spectrum of organisms, from unicellular organisms such as *Paramerium* and *Sacrharomyfiei,* to numerous tissues in mammals. The wide distribution indicates that this class of ion channel is important and is originated early in the evolution of eukaryotes.

Table 5.1 The Distribution of Ca^{2+}-Dependent K^+ Channels
(Hinirchsen RD In: Calcium-Dependent Potassium Channels. R.G. Landes Company, Austin, 1993)

Organism	Tissue or Location
Rat	Skeletal Muscle
	Smooth Muscle
	Brain
	Cerebral Cortex
	Ant. Pituitary
	Pancreatic 5 cells
	Hippocampal Neurons
	Sympathetic Neurons
	Retinal Amacrine Cells
	Thymic Lymphocytes
	Myometrium
	Glioma C6 Cells
	Pulmonary Artery
	Tumor Mast Cells
	Cortical Coll. Ducts
	Neurohypophysis
Rabbit	T-Tubules
	Smooth Muscle
	Renal Collecting Ducts
	Renal Brush-Border Cells
	Colonocytes
	Portal Vein
	Colon Epithelial cells
Mouse	Lac. Gland Acinar Cells
	Fibroblasts
	Macrophage
	HIT cells
	Spinal Cord Neurons
	Neuroblastoma Cells
	Pancreatic D Cells
	Ant. Pituitary Cells
	Motoneurons
	Parotid Acini
	Olfactory Neurons
Toadfish	Saccular Hair Cells
Guinea Pig	Trachea Cells
	Hepatocytes
	Smooth Muscle
	Intestinal Ileum
	Sensory Neurons
	Hippocampal Neurons
	Pancreatic Acinar Cells
Frog	Semicircular Canals
	Eye Lens
	Esophagus
	Neuromuscular Junction
	Smooth Muscle
Bovine	Endothelial Cells
	Chromaffin Cells
	Smooth Muscle
Canine	Kidney
	Smooth Muscle
	Mesenteric Artery Cardiac Muscle
	Smooth Muscle
	Coronary Artery
Human	Chondrocytes
	Sensory Neurons
	Leukemic T Cells
	Smooth Muscle Cells
	Ciliary Epithelium Cells
	Ant. Pituitary Cells
	Platelets
	Osteosarcoma Cells
	Liver Cells
	Eccrine Sweat Glands
	Breast Carcinoma
	Pancreatic Cells
	Promyelocytic Cells
	Colonic Epithelial Cells
	Ehrlich Ascites Tumors
	Glioma Cells
	B Lymphocytes
Hamster	Brown Adipocyte Cells
Porcine	Smooth Muscle Cells
	Pancreas Acinar Cells
	Aortic Endothelial Cells
Ovine	Pituitary Gonadotrophc
Chicken	Osteoblast Cells
Helix	Neurons
	Neuronal U Cells
Aplysia	Pacemaker Neurons
	Neuronal Cells
Euhadra	Neuronal Cells
Amphiuma	Distal Tubule Cells
Shistosoma	Muscle Cells
Yeast	Vacuole
	Plasma Membrane
Paramecium	Cilia and Cell Body
Drosophila	Flight Muscle
	Neuronal Cells

5.2.5 Developmental Regulation of Potassium Channels in Various Tissues

Voltage-dependent potassium channels have a remarkable ubiquity, diversity, and importance in a wide variety of cell types. Recent progress in understanding their susceptibility to modulation by extracellular and intracellular agents and advances in the elucidation of their molecular structure has been obtained (Rudy, 1988; Jan and Jan, 1989). The present focus is prompted by emerging observations that expression of these channels in developing systems is regulated and that functional significance to subsequent differentiation of both rapid signaling capability and expression of other neuronal phenotypes are elucidated.

Voltage-dependent potassium currents differentiate in excitable as well as some classically non-excitable cells from a variety of animals. The development of potassium currents has been studied in a variety of avian and mammalian preparations for which the acquisition of excitability is an early aspect of neuronal differentiation. In several instances, the developmentally regulated expression of prolonged outward potassium currents influences the extent to which sustained inward calcium current contributes to the action potential at early stages of differentiation (Pettigrew et al., 1988; Nerbonne and Gurney, 1989; McCobb et al., 1990). The later acquisition of specific potassium currents is associated not only with the developmentally regulated modulation of excitability but with consequent gains of tissue-specific function. For example, in the chick cochlea, the maturation of a calcium-dependent potassium current permits high-frequency voltage oscillations characteristic of adult hair cells (Fuchs and Sokolowski, 1990).

The program of differentiation of outward current in Drosophila flight muscle differs from that in amphibian myocytes (Salkoff, 1985). The first current to appear is I_K A, and this current is affected by mutations in the Shaker gene. A sustained outward current develops subsequently, and finally a transient calcium-dependent outward

potassium current is acquired only after I_{Ca} is present. It is not known whether the calcium dependent potassium channels are present at earlier times and not functional because of the absence of I_{Ca}, or if I_{Ca} and I_K C differentiate with a similar time course.

Cardiac ventricular muscle has an inward rectifier potassium current that increases with development (Josephson and Sperelakis, 1990), and I_K, becomes larger in aortic smooth muscle (Bregestovski et al., 1988). Estrogen stimulation of rat myometrial smooth muscle cells causes an increase in levels of mRNA encoding voltage-dependent potassium channel (Boyle et al., 1987), that presumably occurs periodically *in vivo.*

Similarly, the cells of the immune system present an interesting complexity of voltage-dependent channels that is regulated initially by development. Later the appearance of voltage-dependent potassium channels is influenced by such processes as antigen recognition and substrate adherence (McKinnon and Ceredig, 1986; Lewis and Cahalan, 1988 a & b). The complement of potassium channels present in T lymphocytes is indicative of its age and function. In addition, just as for neuronal potassium channels, intracellular messengers mediate the modulation of these channels in lymphocytes.

The maturation of voltage-dependent potassium currents has been investigated in cleavage-stage embryos (Takahashi and Yoshii, 1981; Hirano et al., 1984; Hirano and Takahashi, 1987; Block and Moody, 1987; Okado and Takahashi, 1988, 1990asb; Simoncini et al., 1988) as well as in terminally differentiated tissues. Development of ionic currents in embryonic cells that are still mitotically active proceeds along lineage-specific as well as non-lineage-dependent pathways.

During embryogenesis of the ascidian *Boltenia*, a sodium current is lost in all cells by the eighth-cell stage (Block and Moody, 1987). A delayed outward potassium current is acquired by all cells approximately 3 hr after gastrulation; in contrast, a calcium

current is acquired only by cells of a muscle lineage (Simoncini et al., 1988). Early embryogenesis in Boltenia can thus be divided into a two-phase process. During the first phase, starting at fertilization and extending to just after gastrulation, currents present in the oocyte are lost to give rise to blastomeres that have similar electrical characteristics; during the subsequent phase, cells of a muscle lineage acquire new and specific currents.

In summary, the acquisition of voltage-dependent potassium currents that are characteristic of mature cells is developmentally regulated in embryonic cells; their initial expression is observed in cells that are mitotically active as well as ones that are postmitotic and undergoing terminal differentiation. In most instances, the program that directs the schedule for potassium current expression is a major determinant of the general properties of electrical excitability of these differentiating cells.

5.2.7 Potassium Channel in Cultured Chondrocytes

Very little is known on the electrophysiological properties of chondrocytes. Using conventional intracellular techniques, it was shown that during differentiation, the resting membrane potential in chondrocytes of the rabbit epiphysial plate changes, along with the potassium intracellular activity (Edelman et al., 1985). Ionic currents could be very important in the maturation of the chondrocytes and other cells. However, no further information is available on the ionic mechanism involved in the regulation of the membrane properties until 1990's.

Chondrocytes obtained from resting zone cartilage of pig scapulas, were first studied by Grandolfo and his co-workers (1990) with patch clamp technique in cell-attached recording configuration, and single potassium channels were then characterized at

different stages of culture. After 3 days of culture, outward currents were present, with an open probability increasing with depolarization, and the K^+ channels showing a mean slope conductance of 82 pS in asymmetric and 168 pS in symmetric potassium solution. Tetraethylammonium (TEA) and quinidine blocked the channels. Cells at confluence showed similar channel activity, with conductances of 121 and 252 pS, respectively. They concluded that culture time and/or conditions may modify K^+ channels or induce the expression of a new type of channels. Two years later, Grandolfo et al. (1992) reported that, in chondrocytes, a class of Ca^{2+}-activated K^+ channels is present and their activity is related to an increase of $[Ca^{2+}]_i$.

The effect of propofol on the voltage-activated potassium channels in pig articular chondrocytes was investigated (Mozrzymas et al., 1994). Propofol, a widely used general anaethetic, exerts a scavenging effect against free radicals in artificial synovial fluid which was found to reversibly block the potassium channels in a dose-dependent manner. The blocking effect was voltage-independent. Interestingly, half-blocking concentration found in their experiments was also smaller than the blood concentration of propofol used in anaesthesia. These results show that propofol may strongly affect the potassium channels in some non-excitable cells.

With the use of the whole cell arrangement of the patch-clamp technique, an outward-directed time-dependent potassium current was identified in cultured chicken growth plate chondrocytes (Walsh et al., 1992) This delayed rectified potassium current (I_K) activated with a sigmoidal time course during voltage steps to potentials positive to -40 mV. The half-maximal voltage required for current activation was determined to be -8 mV. The reversal potential (E_{rev}) for I_K, measured using deactivating tail currents, was -72 mV in the presence of 140 mM internal and 5 mM external $[K^+]$ solutions. Changes in external $[K^+]$ caused E_{rev} to shift in a manner expected for a potassium-selective channel. In addition, increasing external $[K^+]$ from 5 to 50 mM caused the slope conductance of the tail currents to increase twofold. The chondrocyte I_K was inhibited by the potassium-channel blocker 4-aminopyridine (4-AP) and by the scorpion venom toxin charybdotoxin (CTX) but

was unaffected by tetraethylammonium (TEA). Addition of 20 μM $ZnCl_2$ reduced I_K in a voltage-dependent manner with the greatest inhibition found to occur at potentials near the threshold for current activation. Reduction of I_K by $ZnCl_2$ was accompanied by a slowing in the kinetics of I_K activation. On the basis of the gating and pharmacological properties of this current, it is suggested that the chondrocyte channel belongs to a superfamily of $[K^+]$ channels found in bone and immune system cells. The chondrocyte $[K^+]$ channel may contribute to the unusually high $[K^+]$ found in the extracellular fluid of growth plate cartilage.

Single channel K^+ currents were also recorded from inside-out patches of membrane obtained from cultured chicken growth plate chondrocytes (Kathryn et al., 1994). With a symmetrical 140 mM concentration of K^+ across the cell membrane, a large conductance (211 pS), outward-rectifying, K^+ channel was identified. Opening of this channel was dependent on the presence of internal Ca^{2+} with half maximal activation of the channel occurring with 3 mM Ca^{2+}. The channel was blocked by internal application of TEA (IC_{50} = 45 mM) and 1 mM Ba^{2+}. Application of the catalytic subunit of protein kinase A to the cytosolic membrane resulted in over a three-fold increase in the open probability for the channel. Thus, growth plate chondrocytes contain a large-conductance, Ca^{2+}-activated K^+ channel which can be up-regulated during stimulation of protein kinase A.

Besides the pig and chicken chondrocyte model, two types of potassium channels in horse articular cartilage were identified by patch clamp experiments in the cell-attached configuration, one characterized by a conductance of 40 pS and the other of 100 pS. No active K^+ channels were found at Vpip = 0 (Vittur et al., 1994).

Up to now, all the studies on K^+ channel of chondrocytes were in cell culture model. It was demonstrated that culture time and/or conditions may modify K^+ channels or induce the expression of a new type of channels (Grandolfo et al., 1990). It is necessary to establish an *in situ* growth plate model for further characterization of the existence and properties of various types of potassium channel in chondrocytes.

Practically, growth plate chondrocytes refer to a mixture of chondrocytes in various differentiative (resting, proliferative and hypertrophic) stages. These cells will further differentiate or de-differentiate in an artificial culture condition. In order to investigate the real characteristics of K^+ channel in growth plate chondrocytes, an *in situ* growth plate model is essential. That is the aim of this study.

5.3 METHODS

5.3.1 Growth Plate Cartilage Slice Preparation

Pigs between 4 - 5 weeks of age (about 10 kg) of both sexes were used in this study. After the animal was sacrificed by injecting 10 ml of 2.5% (w/v) pentobarbital directly into its heart, the rib cage was aseptically dissected and cleaned until free from soft tissue. The growth plate cartilage was taken out at the osteochondral junctions of all the ribs. The growth plate block was then placed in DMEM (Sigma, St. Louis, Missouri).

Thin coronal longitudinal slices (250 μm) containing different phases of differentiation were sectioned using a vibrating microtome (Campdem Instruments Ltd., U.K.). The physiological saline for sectioning and for subsequent recordings consists of the followings (in mM): NaCl, 125; KCl, 2.5; $MgSO_4$, 1.2; $CaCl_2$, 0 or 2.5; NaH_2PO_4, 2; glucose, 11.5 and $NaHCO_3$, 26, which was continuously bubbled with 95% O_2 and 5% CO_2. The slices were then treated with digestion solution containing 0.35% (w/v) collagenase (Type IIA) and 0.5% (w/v) hyaluronidase (Type I-S) in DMEM and maintained at a temperature of 37°C for at least 3 hours before experiment.

5.3.2 Electrophysiological Recordings

During an experiment, one piece of enzyme-digested cartilage was transferred to a small volume (0.8ml) superfusion chamber that was mounted on an upright microscope (Zeiss Axioskop). The slice was weighed down by a nylon grid and was superfused with the physiological saline continuously at room temperature (20 - 25°C) at a rate of 1.5 to 2 ml/min. To visualize the chondrocytes, a combination of differential interference contrast optics and infra-red video microscopy was adapted

from the method described by Stuart et al. (1993). Briefly, the cells were viewed through the eyepiece using the differential interference contrast system of the microscope. To increase the contrast, the image was projected into a video camera mounted onto one of the documentation ports. The camera used is one which is sensitive in the infra-red range (Hammatsu C2700-07). To observe cells in the deeper layer within the slice, an infra-red filter was inserted into the lightpath between the halogen lamp (for brightfield illumination) and the condensor. However, this feature was not always required during an experiment. With this arrangement, chondrocytes of different differentiation stages can be clearly identified according to their sizes and positions within the slice.

Whole-cell patch-clamp recordings from different types of chondrocytes were obtained using an Axoclamp-2B amplifier (Axon Instruments). Whole-cell pipettes typically had resistances of 4-6 MΩ when filled with internal solutions of the following composition (in mM): KCl 140; EGTA 1.0; $CaCl_2$ 1.0; $MgCl_2$ 1.0; Na_2-ATP 2.0; HEPES 10 and pH adjusted to 7.3 with KOH. Monitoring through a television connected to the camera, a pipette was placed on the cell body of the chondrocytes (Figure 5.4). Tight seal (> 1GΩ) was made, followed by 'breaking in' and the voltage was clamped using the continuous single electrode voltage clamp mode. No series resistance compensation was made but the cells were rejected if the series resistance increased significantly during recording. Current signals were filtered at 1 kHz and were stored in a DAT recorder (SONY) modified for recording AC and DC signals at 32kHz. Voltage-pulses were generated on line by the Voltage-clamp and Patch Software (Cambridge Electronic Design, CED) using the A/D D/A converter CED 1401 Plus which also digitized the current responses and saved into computer files for later off-line analysis.

Chemicals

The chemicals used in the present study were all obtained from Sigma. The data are expressed as mean ± S.E.M. The statistical test used were stated in the appropriate sections.

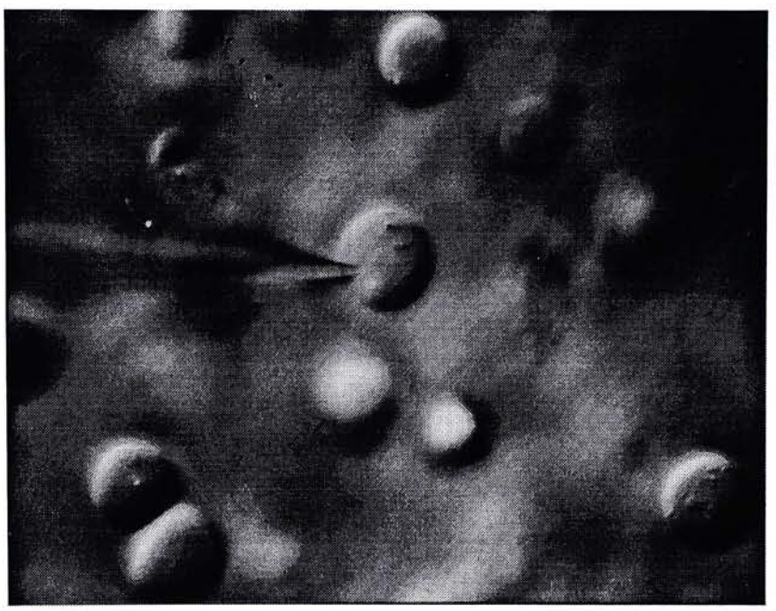


Figure 5.4 Patching of porcine costal growth plate chondrocyte at hypertrophic zone for whole cell patch clamp analysis. (60x)

5.4 RESULTS

5.4.1 *In Situ* Partial Digestion of Growth Plate Chondrocytes for Patch Clamp Study on Membrane Currents

In growth plate cartilage, chondrocytes in various differentiation states are located in an organized manner (Refer to Chapter 2, Figure 2.1a). It is easy to identify the resting, proliferative or hypertrophic chondrocytes by their cell morphology and relative position. However, when the cells were released from cartilage matrix by enzyme digestion, a heterogeneous mixture of chondrocytes in different maturation stages were obtained (Refer to Chapter 2, Figure 2.2a). Thus, chondrocytes in various stages are no longer identifiable.

In this study, longitudinal slices of growth plates were obtained by sectioning with a vibrating microtome. The cell morphology and relative position were preserved (Figure 5.5a) although many cells at the cutting edge were unavoidably damaged. Since the intact chondrocytes, even those near the surface, were covered with a layer of hard cartilage matrix of collagen and proteoglycan, the patch pipette could not make good contact with the cell membrane and electrophysiological recording could not be performed. In order to remove the covering matrix and maintain the relative cell position, partial enzyme digestion with different concentrations of collagenase and hyalruonidase for different durations were tested. In the preliminary studies, different combinations of enzyme concentration, temperature and duration of digestion were tested. We finally arrived at the following protocol which routinely produced partially digested growth plates with cells at the surface that were free of extracellular matrix and suitable for patch clamp recordings. The optimal conditions are at least 3 hours of treatment at 37°C with 0.35% collagenase and 0.5% hyaluronidase. As can be seen in Figure 5.5b, although the cells become spherical after partial digestion of the cartilage, the chondrocytes still showed a healthy appearance. The hypertrophic cells could be easily identified by their large cell size

(Table 5.2). In addition, the relative locations and intercellular distances were preserved that allowed us to identify chondrocytes in all the three stages of differentiation. Without this treatment, it was virtually impossible to patch the cells while after digestion the average rate for tight seal formation was about 40%. It was also found that hypertrophic cells had a shorter survival period (about 3 - 4 hours) while the resting and the proliferative had a longer survival period (up to 6 hours) during the digestion.

Table 5.2 Cell size of chondrocytes in various cartilage zones (Mean ± S.E.M.)

	Diameter (μm)	Surface area (μm^2)	n
Resting zone	12.5 ± 0.4	449.3 ± 58.5	9
Proliferative zone	13.2 ± 0.5	598.7 ± 65.3	8
Hypertrophic zone	20.1 ± 0.8	1286.0 ± 96.5	9

5.4.2 Whole-cell Outward Currents in Growth Plate Chondrocytes

Figure 5.6a shows an example of the whole-cell membrane current of a chondrocyte that can be activated with a standard protocol of 300ms depolarisation which steps from a holding potential of -80mV. There was no active current evoked in response to a hyperpolarisation. However, when the membrane potential was stepped beyond -40mV, a large outward current was activated. With increased depolarization, both the rate of activation and the amplitude of the currents increased. The increases in currents between each step became smaller during voltage step potentials ranging from -40 to +20 mV and became larger from +20 to +60mV. During prolonged depolarizations (e.g. longer than 200 ms) the current showed sign of inactivation. Such decline was more prominent in the more depolarized potential (> +20mV).

It was also observed that the amplitude of the outward current was dependent on extracellular calcium ion concentration. When the normal 2.5mM extracellular $CaCl_2$ was removed and replaced with 10mM of $MgCl_2$, the magnitude of the outward current decreased especially at the more depolarised voltage range (Figure 5.6b). These data suggest that the outward current consist of a Ca^{2+}-dependent and a Ca^{2+}-independent component.

The above observations on the behavior of outward currents are similar in chondrocytes of all three different differentiation states. Therefore their characteristics are discussed below without reference to a particular cell type. However, the total magnitude of the outward currents and the relative contributions of the two outward current components differ among cell subpopulations. These findings will be summarized in the last section (Section 5.4.5).

5.4.3 Properties of Ca^{2+}-Independent Outward Current in Growth Plate Chondrocytes

As the Ca^{2+}-dependent component of the outward current was activated predominantly at higher (>40mV) membrane potentials *(detail discussion refer to section 5.4.4 and Figure 5.11)* and in the presence of extracellular calcium, the behavior of the calcium-independent component described below was obtained either in the absence of extracellular calcium or analyzed up to the membrane potential <40mV. Figure 5.7a shows a typical example of such a recording. In the absence of calcium and with a standard depolarization protocol, the outward current activated, following a delay, when the membrane potential was depolarized beyond -40 mV. With increased depolarization, both the rate of activation and the amplitude of the currents increased. This outward current was present in every chondrocyte successfully examined (total of 55 cells).

In Figure 5.7b the normalized conductance for the potassium current displayed in Figure 5.7a is plotted as a function of the test voltage. The half maximal voltage ($V_{1/2}$) required for activation was -30 mV in this experiment. Overall, in 9 cells examined, $V_{1/2}$ was determined to be -25 ± 3 mV.

In order to test whether the outward current represents K^+-efflux, the reversal potential of the deactivating tail currents was determined. This was achieved by stepping the membrane from -80mV to 20mV at which a large portion of the calcium-independent channels were activated. This is followed by stepping back to different membrane potentials from -60 to -120mV. The point at which the tail current becomes zero was the reversal potential. The response to this stimulus protocol is shown in Figure 5.8a. By plotting the current-voltage relationship of the tail current (Figure 5.8b), the reversal potential of the current of this cell was found to be -85mV, which is typical of 6 cells from different differentiation stages (mean -88 ± 6mV). This is more positive than the calculated Nernst equilibrium potential (E_k) of -95 mV for a K^+-selective channel under these conditions, suggesting that there may be some finite permeability to other ions or contamination by the calcium-dependent component which may have a different reversal potential *(see Section 5.4.4)*. The tail current also indicates that the ionic channels underlying this outward current shows an outward rectifying characteristic.

To confirm the K^+-selectivity of this outward current, the K^+-channel blocker tetraethylammonia chloride (TEA) was used. Twenty mM of TEA largely abolished the outward current activated at 40mV and the effect was partially washable. A typical example of the effect of TEA is shown in Figure 5.9. The effect of TEA was compared in the three different stages of chondrocytes. TEA exerted similar effects on the Ca^{2+}-independent outward current in all three stages of chondrocytes (Figure 5.10).

Thus, with respect to the time and voltage dependency, the ionic selectivity and pharmacology, the Ca^{2+}-independent outward current is similar to the delayed K^+

rectifying current found in nerve and muscle. This current will be referred to as the chondrocyte delayed rectifier K^+ current (I_k).

5.4.4 Properties of Ca^{2+}-dependent Outward Currents in Growth Plate Chondrocytes

Figure 5.11a shows the current-voltage relationship of the whole-cell outward currents in a typical hypertrophic chondrocyte in normal extracellular calcium (2.5mM) and in Ca^{2+}-free solution. When the membrane potential is more negative than -40mV, these two currents are almost identical. However, in more depolarized membrane potential, especially >40 mV, the total outward current is significantly higher when there is 2.5mM of extracellular Ca^{2+}. When subtracting the Ca^{2+}-independent current from the total control current, the Ca^{2+}-dependent current is obtained and is shown in Figure 5.11b. It can be seen that the voltage sensitivity for the Ca^{2+}-dependent outward current dramatically increases when the membrane potential is more positive than 20mV. Similar results were also obtained from proliferative cells and resting cells (not shown).

To elucidate the ionic mechanism of this Ca^{2+}-dependent outward current, the reversal potential of the tail current was also studied. This was done by stepping the membrane potential to +80mV before stepping back to different membrane potentials (Figure 5.12a). Although at 80mV both the Ca^{2+}-dependent and Ca^{2+}-independent outward currents are activated, the reversal potential is again close to the equilibrium potential of K^+, at about -85mV (mean -89 ± mV). This indicates that the Ca^{2+}-independent outward current is also K^+ current.

On the other hand, the Ca^{2+}-dependent outward current is more sensitive to TEA when compared with the delayed K^+ rectifier. Figure 5.13 shows that TEA at 20mM abolished 87% of the total outward activated at 80mV (compared with 75% when the test potential was 20mV). Thus, the pharmacological sensitivities of the two K^+-

outward currents are different. However, the pharmacological sensitivities to other K^+-channel blockers were not further characterized in this study.

5.4.5 Comparing the Outward Currents in Chondrocytes of Various Differentiative Zones

Although both the Ca^{2+}-dependent and Ca^{2+}-independent outward currents are expressed in all growth plate chondrocytes examined, the total amount and the relative proportions of these currents are different in different stages of differentiation. Figure 5.14 summarizes the average voltage-current relationships of all three cell types. It can be seen that the resting chondrocytes expressed much lower amount of both Ca^{2+}-dependent and Ca^{2+}-independent outward current. On the other hand, cells from the proliferative and from the hypertrophic zones expressed similar amount of both types of outward currents. The differences between the outward currents of resting cells were compared with those of the other two cell types and were found to be significantly different from -20mV to 80mV (Table 5.3). On the other hand there was little difference between the corresponding values obtained from proliferative and hypertrophic cells.

To determine whether the difference observed simply reflects the change in membrane surface area during differentiation, the current amplitudes were normalized for the cell membrane area. The resulting current density, expressed as pA of current per μm^2, suggests that the proliferative cells expressed more outward current channels per unit membrane area (Figure 5.15). On the other hand, the current densities for resting and hypertrophic cells are similar. This indicates that the number of K^+-channels expressed is not a simple reflection of the membrane surface area but rather some active expression of ionic channels.

The relative proportions of the Ca^{2+}-dependent and Ca^{2+}-independent components were also studied for different cell types. The remaining outward currents activated at

+80mV in Ca^{2+}-free solutions are expressed as percentage of the control and summarized in Figure 5.16. It can be seen that resting cells expressed a relatively higher percentage of Ca^{2+}-dependent outward currents, about 60%. On the contrary, the Ca^{2+}-dependent components of outward currents in the proliferative cells and the hypertrophic cells are about 40%. By simple calculation, the Ca^{2+}-dependent outward current is higher in resting cells, about 60%, than the proliferative cells and the hypertrophic cells, about 40%.

Table 5.3 The current-voltage relationships of different growth plate chondrocyte subpopulations in response to stimulus in an environment with 2.5mM Ca^{2+}. (* represent statistical significant difference to current of resting chondrocyte with $p < 0.05$)

Membrane Potential (mV)	**Membrane Current (nA)**				
	Hypertrophic chondrocytes		Resting chondrocytes		Proliferative chondrocytes
-80	0 ± 0		0 ± 0		0 ± 0
-60	0.111 ± 0.007		0.006 ± 0.005		0.006 ± 0.006
-40	0.028 ± 0.012		0.018 ± 0.012		0.031 ± 0.013
-20	0.161 ± 0.045		0.061 ± 0.020	*	0.179 ± 0.030
0	0.361 ± 0.072		0.139 ± 0.042	*	0.337 ± 0.052
20	0.497 ± 0.088	*	0.220 ± 0.060	*	0.462 ± 0.081
40	0.733 ± 0.126	*	0.378 ± 0.009		0.744 ± 0.182
60	1.139 ± 0.251	*	0.511 ± 0.112		1.031 ± 0.304
80	1.841 ± 0.4778	*	0.667 ± 0.114		1.656 ± 0.561
	(n = 9)		(n = 9)		(n = 8)

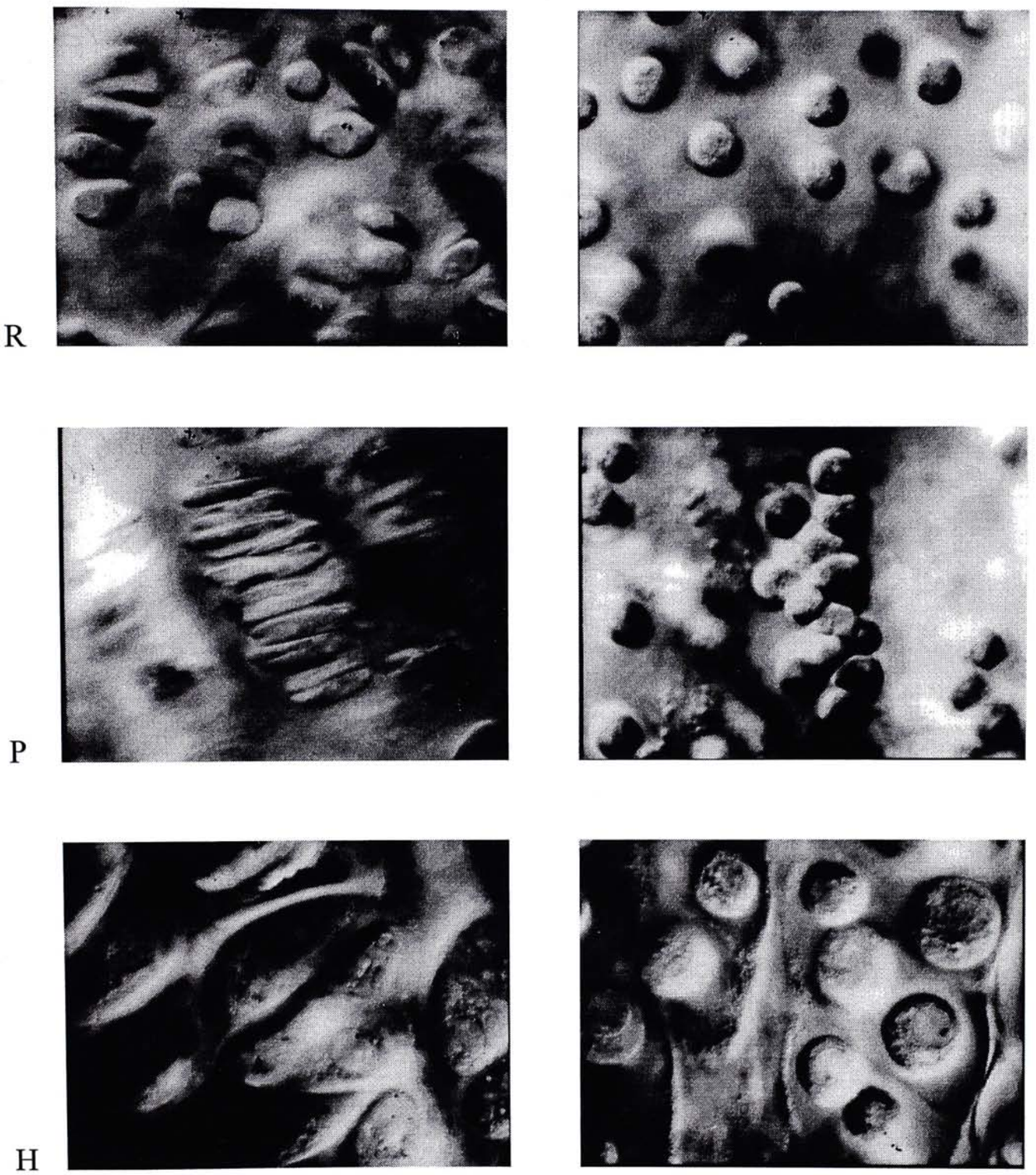


Figure 5.5. Morphology of porcine costal growth plate chondrocyte at resting (R), proliferative (P) and hypertrophic (H) zones before (**a**) and after 3 hrs (**b**) digestion with 0.35% collagenase and 0.5% hyaluronidase. (60x)

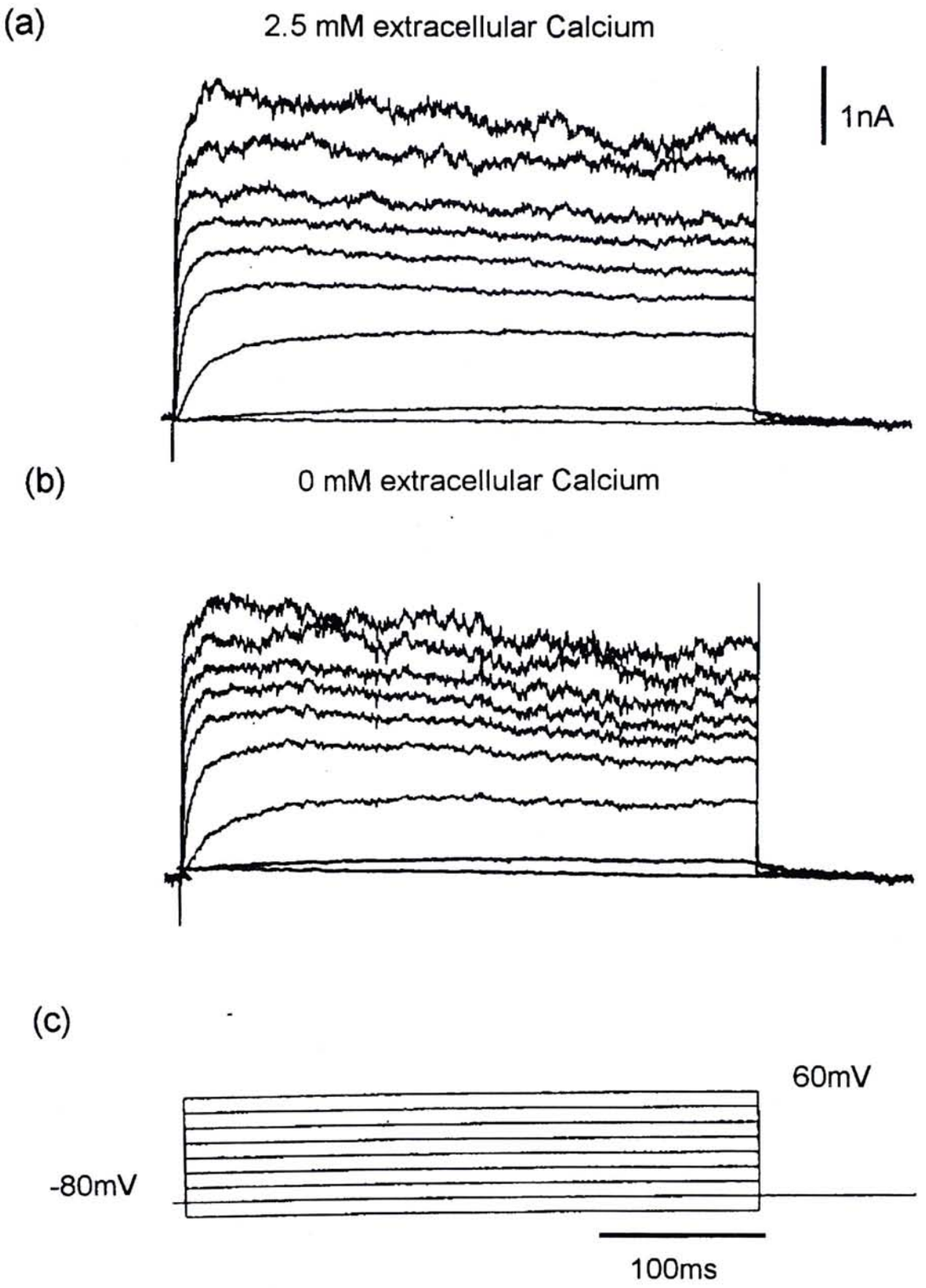
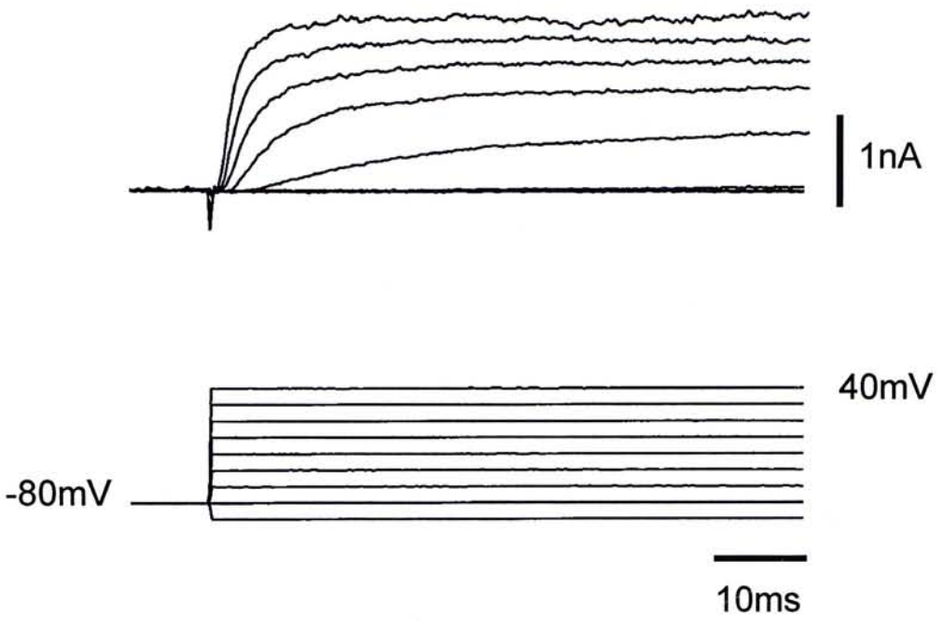


Figure 5.6. (a) In response to the standard voltage step stimulus as shown in (c), a chondrocyte displays an outward current which is both time and voltage-dependent. (b) shows that the outward current is reduced by replacing the extracellular calcium with 10mM of $MgCl_2$. This effect is particularly clear at more depolarised membrane potentials. (c) The voltage step protocol. The time scale applies to all three traces.

(a)



(b)

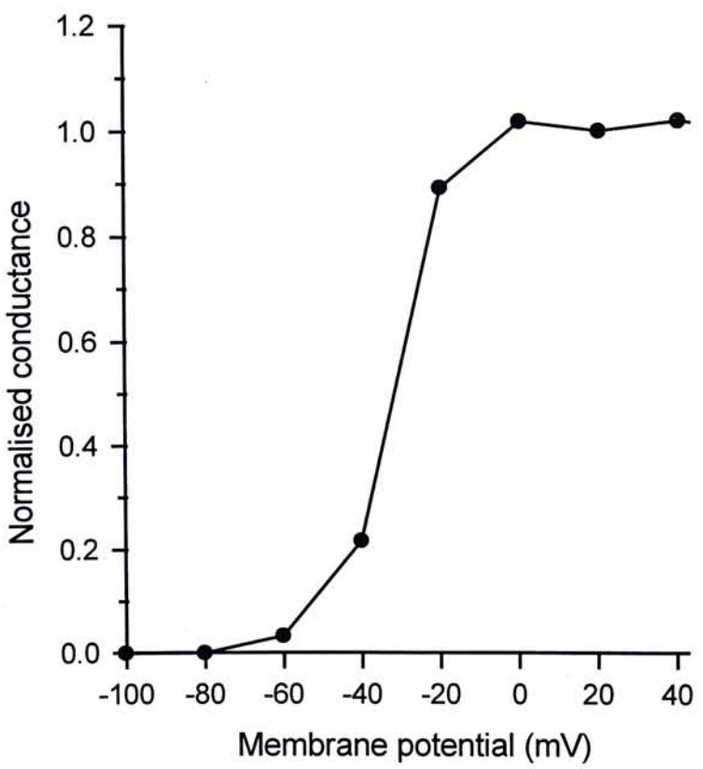


Figure 5.7 (a) At a fast time base, the delayed activation of the Ca^{2+}-independent outward current of a retsing chondrocyte is clearly shown, particularly at less depolarised potential. Stepping to more depolarised membrane potential from the holding potential of -80mV causes a faster activation and a bigger current. (b) When normalised for the driving force ($E-E_k$), the voltage-dependent conductance of the outward current is obtained. In this cell, the half-activation votage is-35mV. *[This is a typical example of 9 trials]*

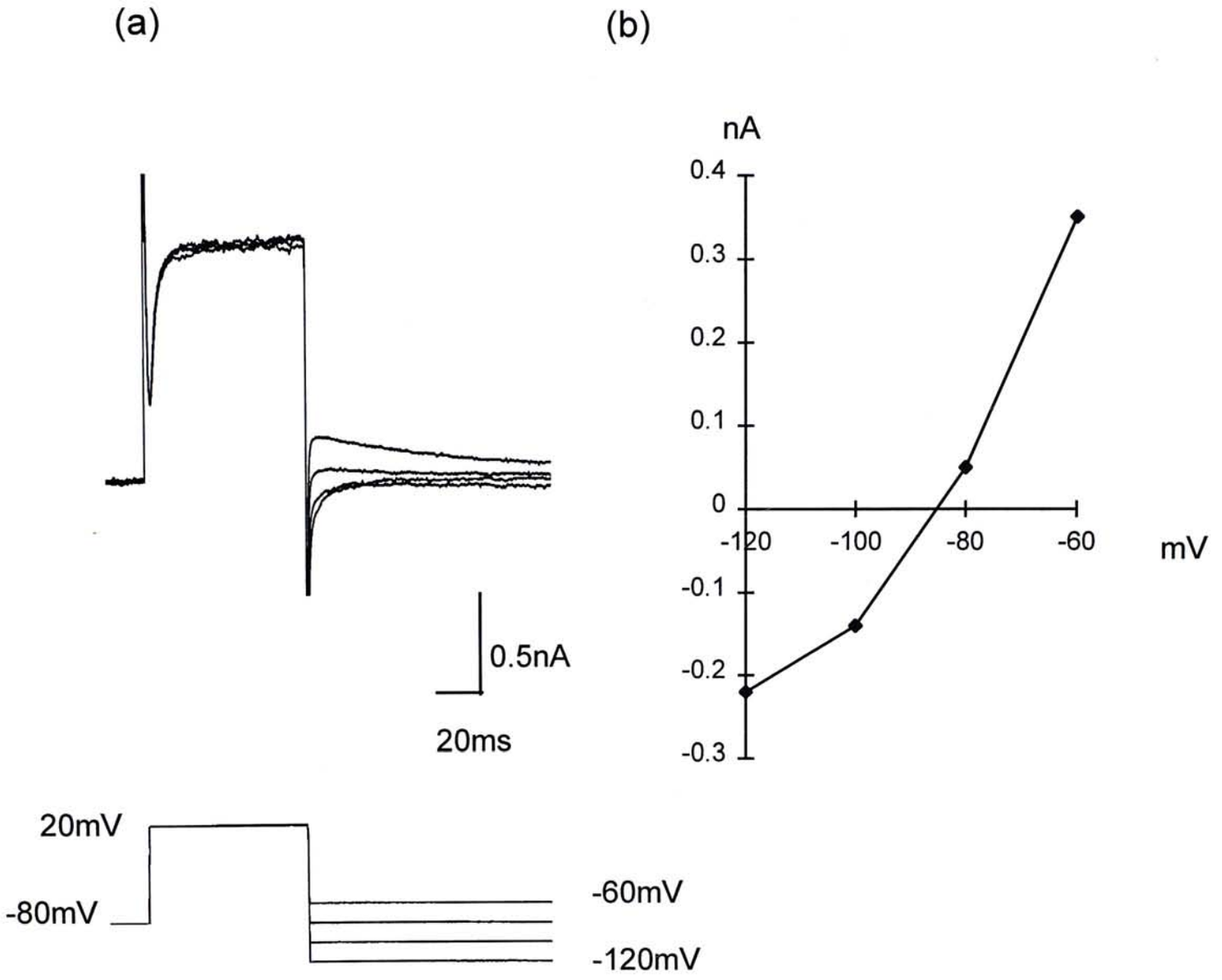


Figure 5.8 (a) The tail current of the Ca^{2+}-independent outward current was evoked by first stepping the membrane potential from -80 mV to 20mV and then stepping back to various membrane potentials between -60 and -120mV. When the membrane potential is more negative than the reversal potential of the permeant ion, the tail current becomes inward going. (b) The tail current magnitudes at 10ms after the depolarising pulse are plotted against the membrane potential. The reversal potential of the tail current of this cell is -85mV. *[This is a typical example of 6 trials]*

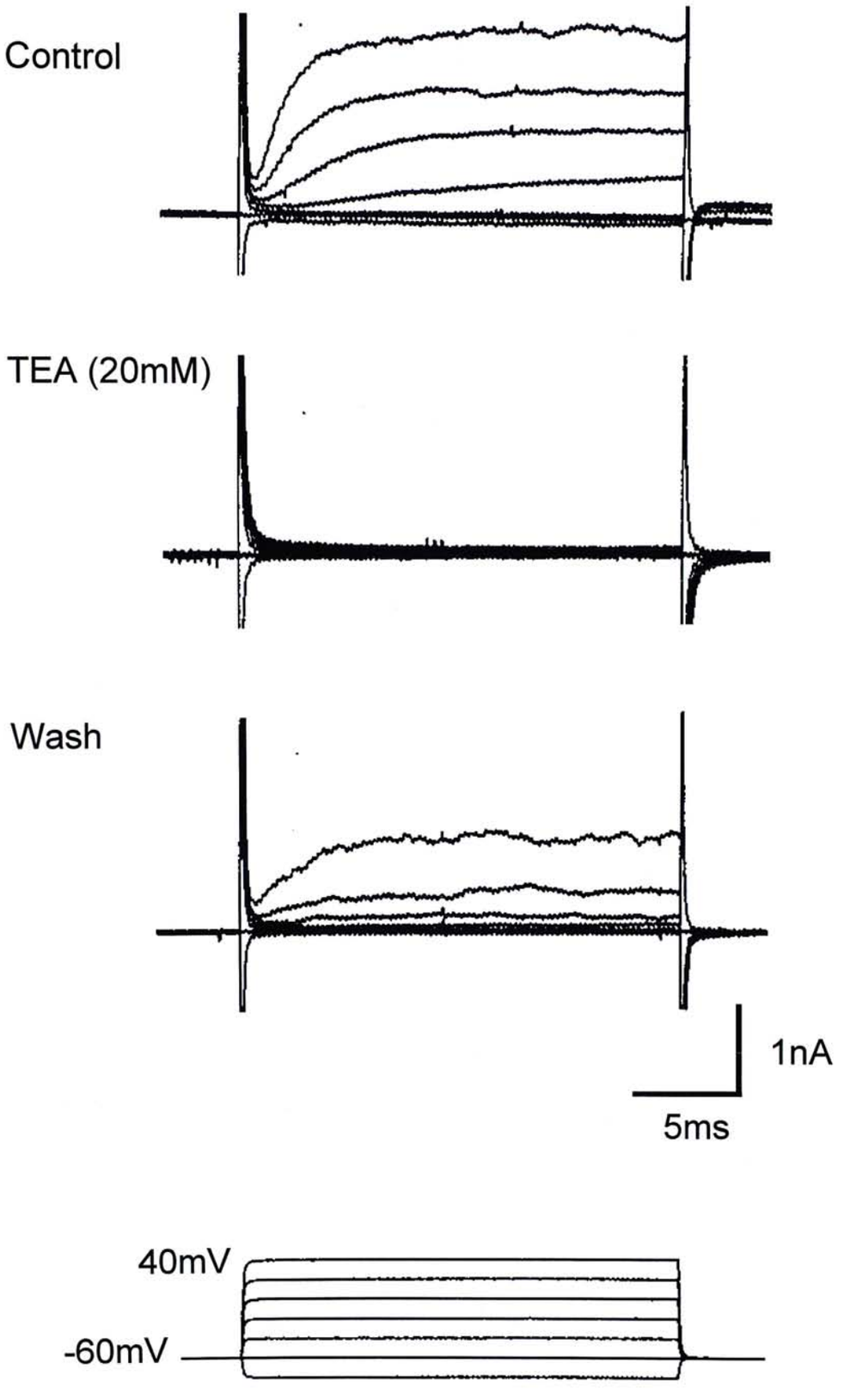


Figure 5.9 20mM of TEA almost completely abolished the outward current activated at 40mV. The outward currents during control, TEA application and washout are shown. The lower trace illustrates the stimulus protocol. No leak subtraction was carried out.

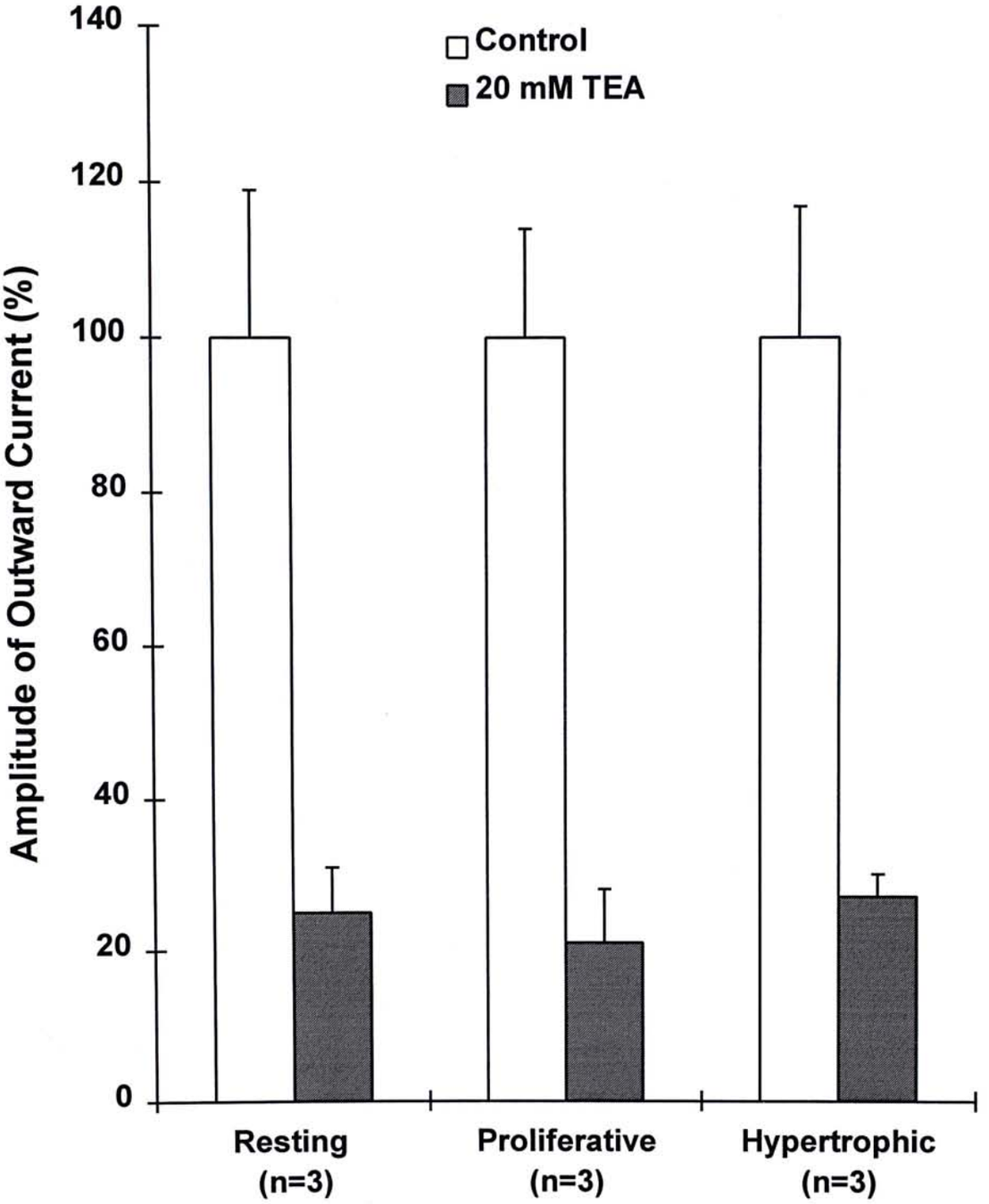
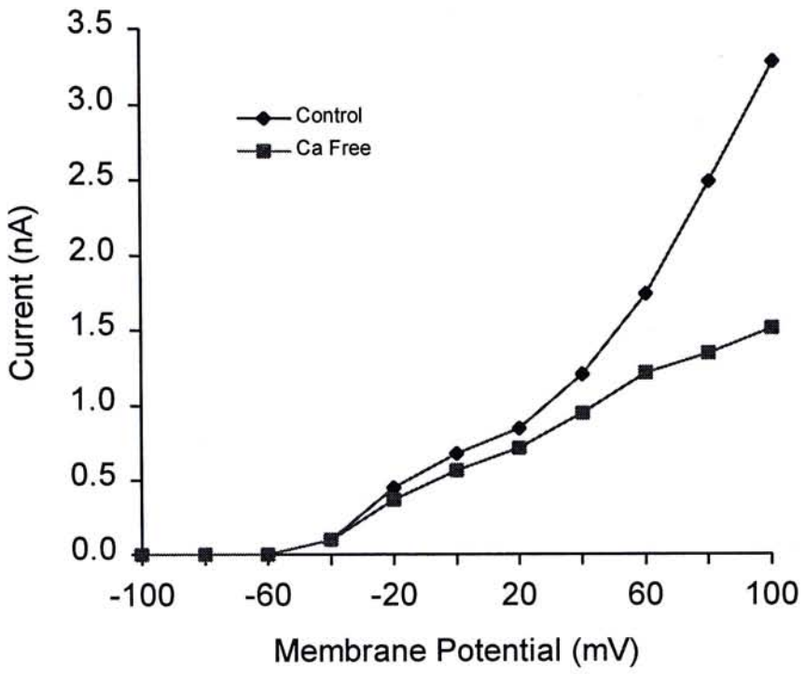


Figure 5.10 The relative inhibition of 20 mM TEA on Ca^{2+} independent outward current of chondrocytes at different differentiation. The 20mM of TEA largely inhibit about 80 -90% of the outward currents activated at 40mV on all the three subpopulations. Data are expressed as mean ± S.E.M.

(a)



(b)

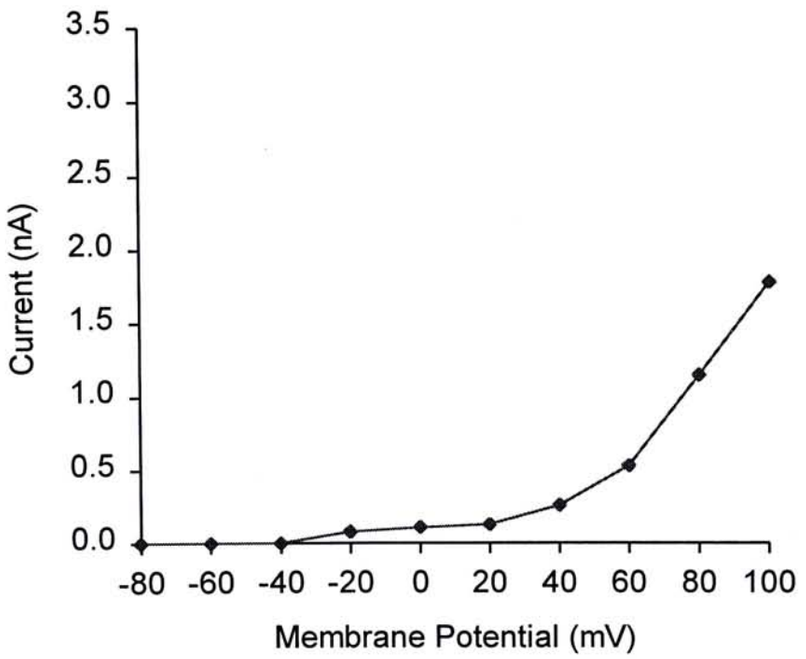


Figure 5.11 (a) The current-voltage relationships of a hypertrophic chondrocyte in response to standard stimulus in control (with 2.5mM Ca^{2+}) and in Ca^{2+}-free environment. In Ca^{2+}-free condition, the outward current is reduced especially at membrane potential more positive than +40mV. (b) When the Ca^{2+}-independent component is subtracted from the total current, the voltage-dependency of the Ca^{2+}-dependent component is revealed.

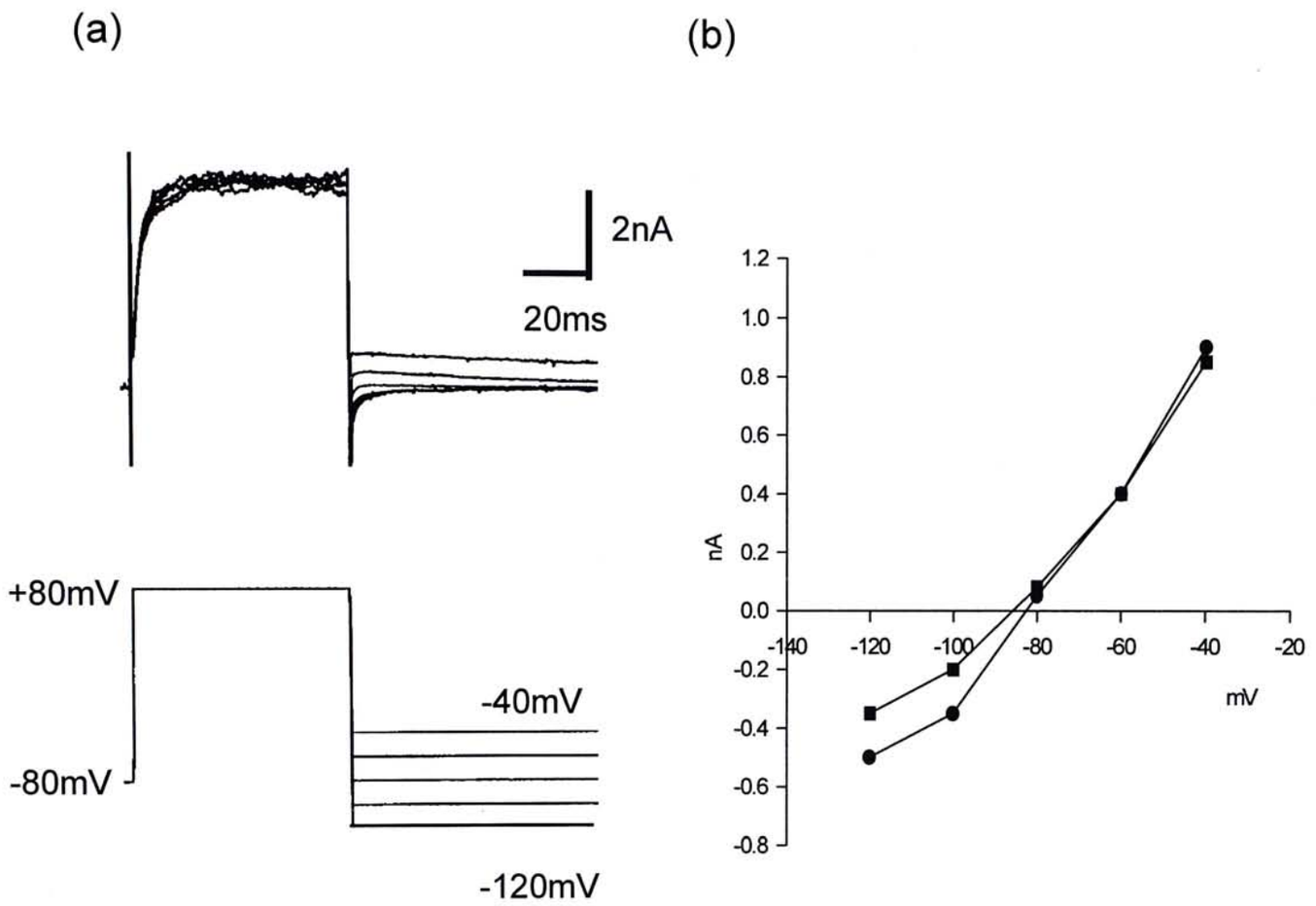


Figure 5.12 (a) The tail current evoked at the end of a depolarization step from -80mV to +80mV. At +80mV both the Ca^{2+}-dependent and Ca^{2+}-independent outward current are activated. (b) illustrates that the reversal potential of the tail current after +80mV activation (closed circle) is very similar to that after depolarisation at +20mV (closed square) indicating that both components of outward current are mediated by similar ion.
[This is a typric example of 5 trials]

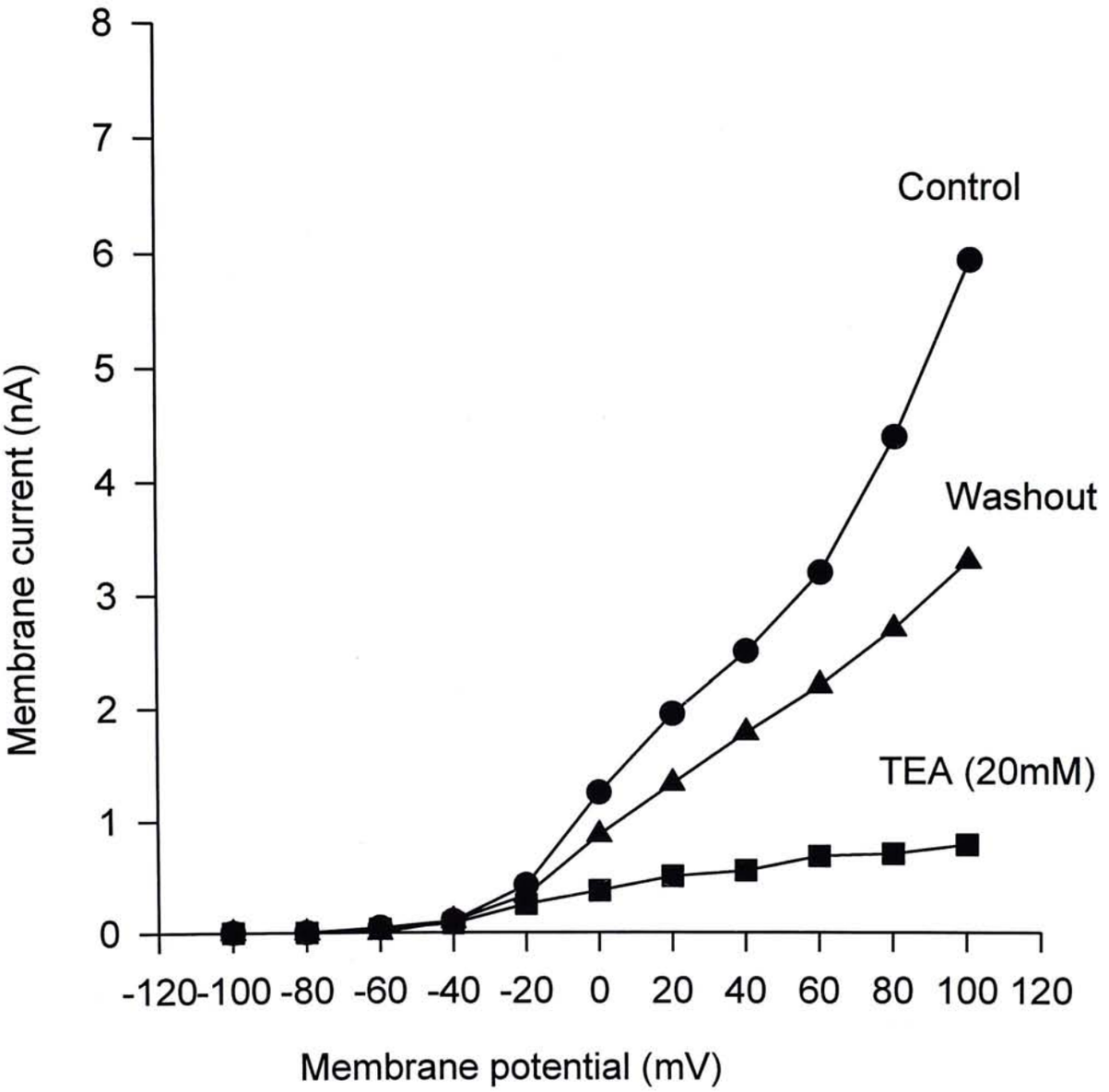


Figure 5.13. Effect of 20mM TEA on the current-voltage relationship of a hypertrophic chondrocyte which clearly possesses both Ca^{2+}-dependent and Ca^{2+}-independent outward currents. TEA decreased the outward current throughout the range of voltage from -20 to +100mV. At +20mV TEA caused a 75% inhibition while at +80mV TEA caused 87% inhibition. The result suggests that the Ca^{2+}-dependent component is more sensitive to TEA.

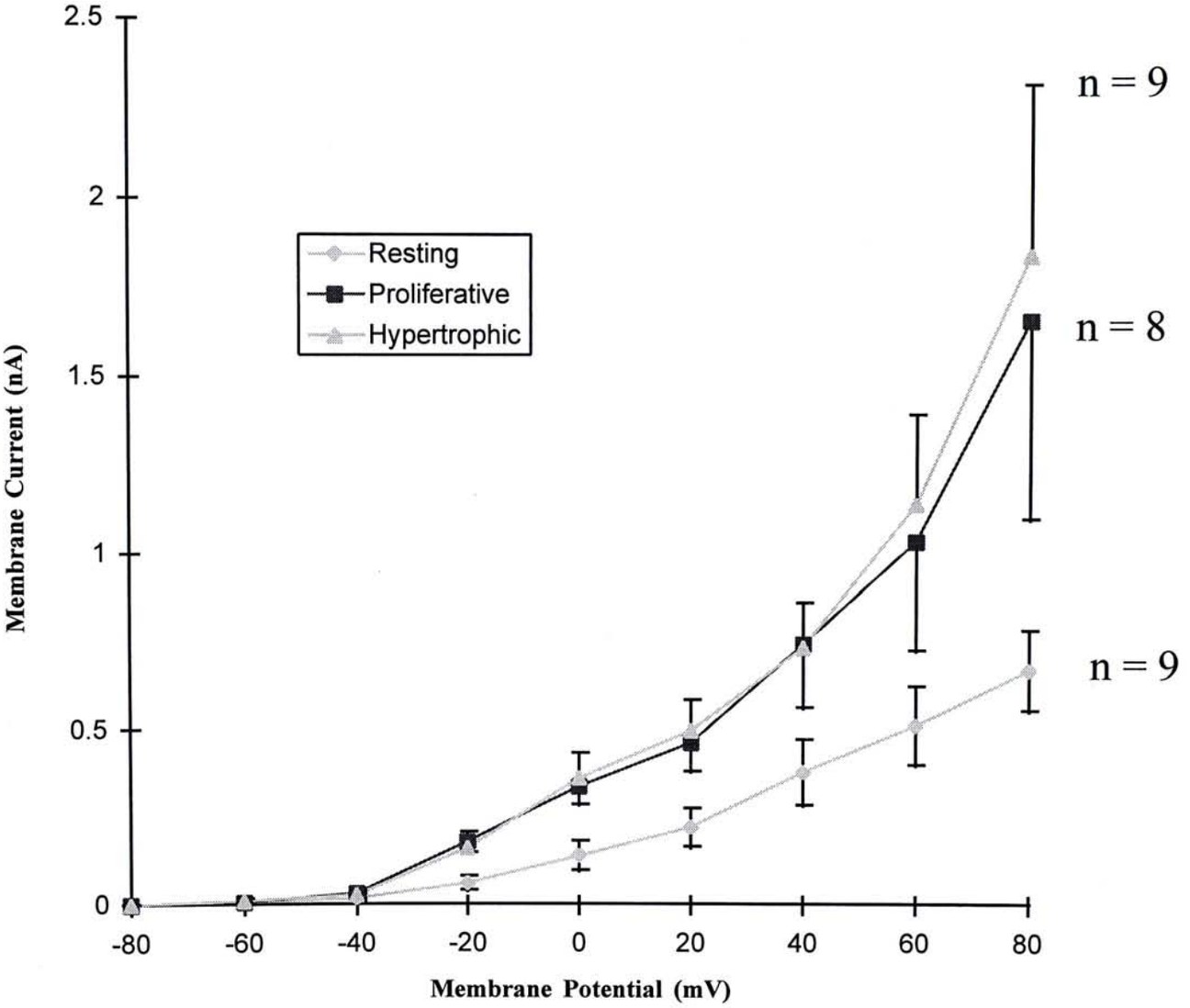


Figure 5.14 Depolarisation-activated outward current in different growth plate chondrocyte subpopulations. The outward currents were evoked in response to standard stimulus protocol in the presence of 2.5 mM extracellular Ca^{2+}. In comparison with the proliferative and hypertrophic cells the resting cells express a significantly smaller outward current for most of the membrane potentials. Data are expressed as mean ± S.E.M.

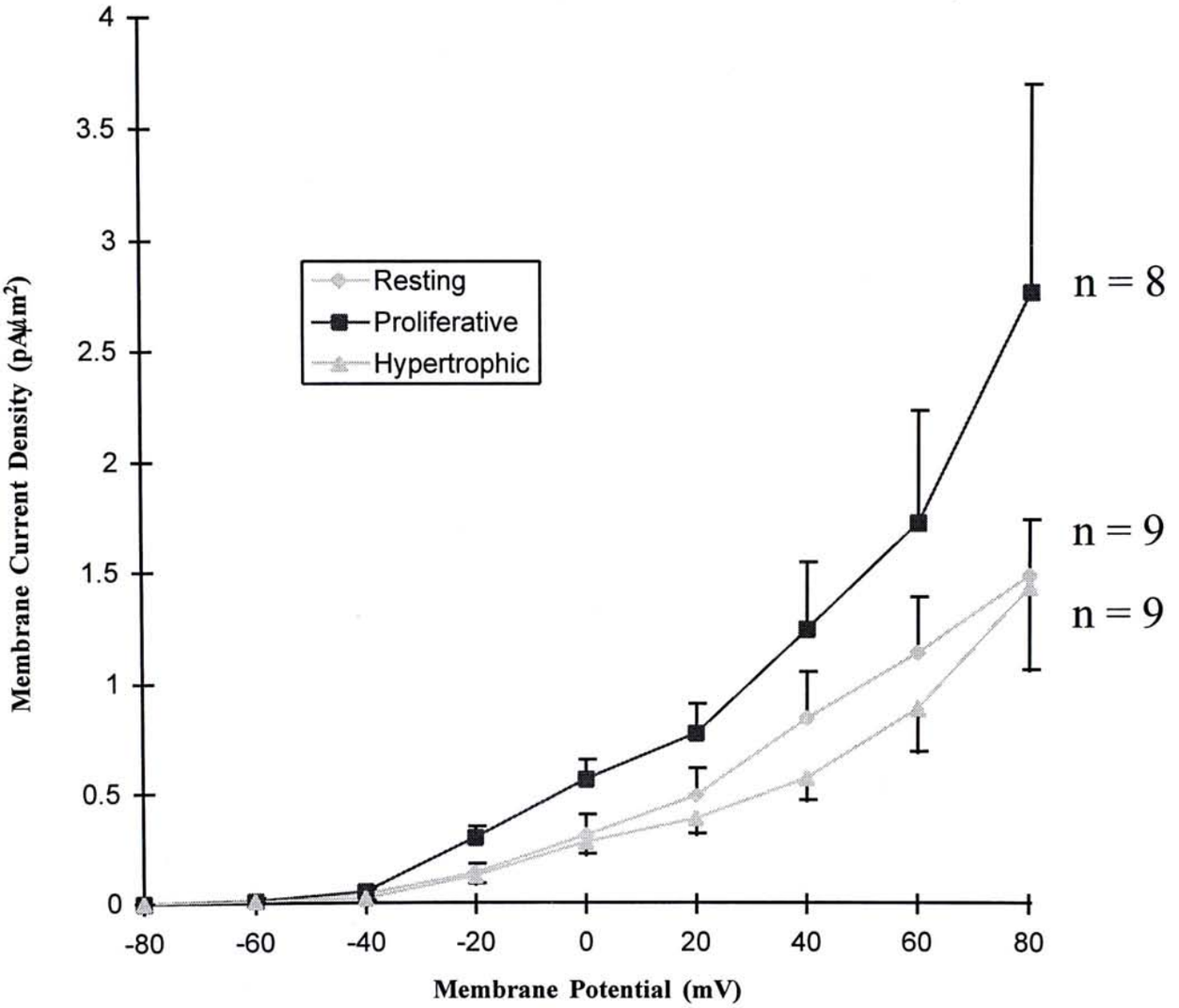


Figure 5.15 Density of depolarisation-activated outward current in different growth plate chondrocyte subpopulations. The outward currents were evoked in response to standard stimulus protocol in the presence of 2.5 mM extracellular Ca^{2+}. The proliferative cells express a significantly higher current per unit cell membrane area. Data are expressed as mean ± S.E.M.

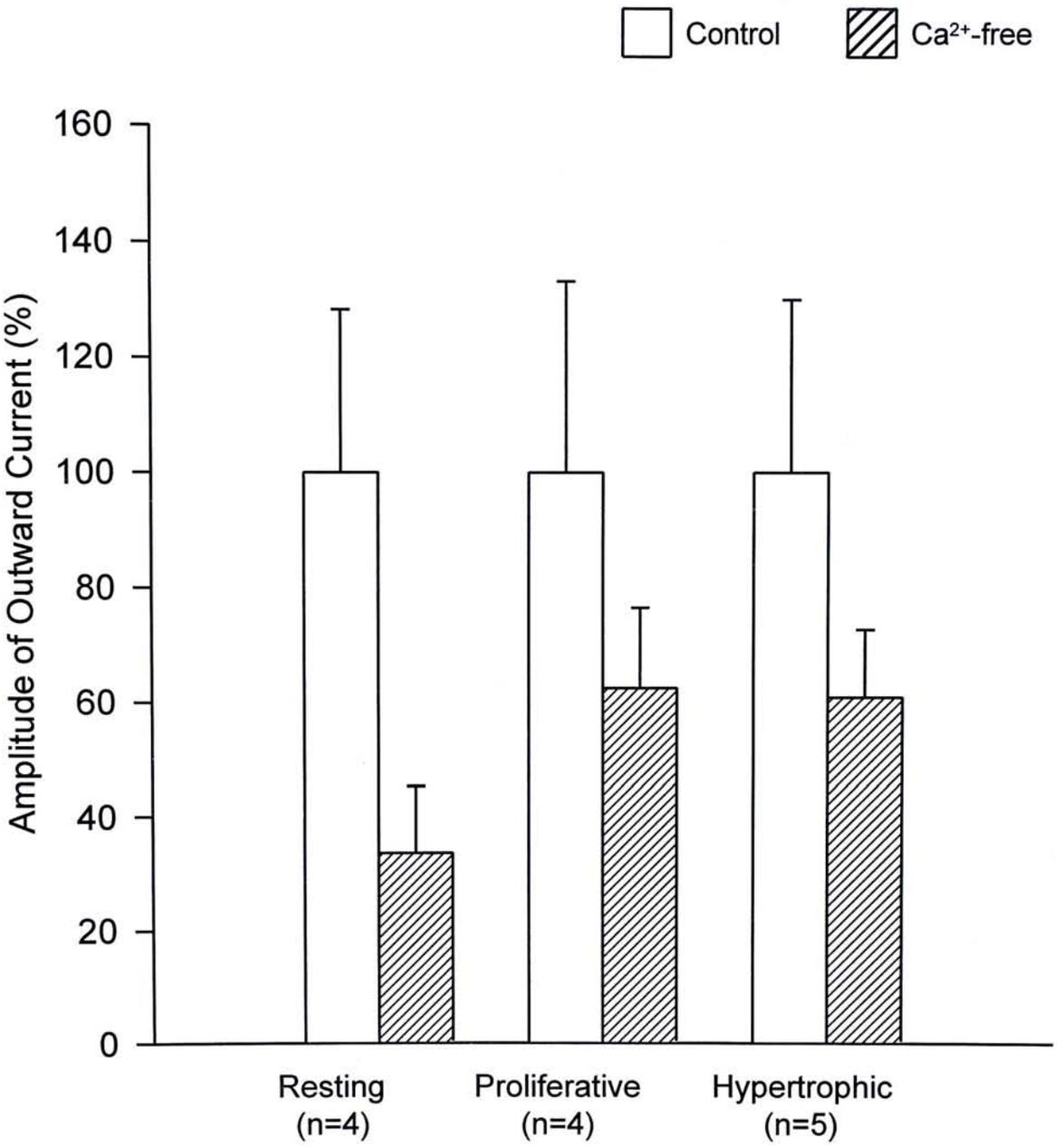


Figure 5.16 The relative proportion of the Ca^{2+}-dependent component of the outward current is determined for chondrocytes at different differentiation stages. The membrane potential was stepped from -80mV to +80mV and the steady state outward current was measured in normal and in Ca^{2+}-free condition. The remaining component in Ca^{2+}-free environment are expressed as percentage of the control current. Data are expressed as mean ± S.E.M.

5.5 DISCUSSION

Growth plate chondrocytes are known to have an active electrolyte metabolism, being involved in the initiation of provisional calcification during bone growth. A characteristic feature of the growth plate is the great increase in cell volume that occurs in the hypertrophic zone and may result from hypotonic swelling. A number of early studies have addressed the question of the levels of electrolytes in both the extracellular fluid (ECF) (Linn and Sokoloff, 1965; Howell et al., 1968; Wuthier,1977) and intracellular fluids (Wuthier and Gore, 1977) of this tissue. ECF from the hypertrophic region of the growth plate of avian species (chicken) has relatively low levels of Na^+ and high levels of K^+ compared with those of blood plasma (Wuthier, 1977). Intracellular fluid (ICF) of chondrocytes isolated from the same region, in contrast, exhibited elevated levels of Na^+ and reduced levels of K^+ compared with typical cells (Wuthier, 1977). The reason for the unusual ECF and ICF levels of K^+ and Na^+ in growth plate cartilage was unclear, although the possibilities of excessive cellular "leakage," or of an inactive Na^+-K^+ pump, were considered. In retrospect, another possible explanation for the high ECF K^+ concentration would be the presence of K^+ channels in the membrane of these chondrocytes. While K^+ channels have been recently studied in some details in cultured osteoblasts (Ypey et al, 1988; Ravesloot et al., 1990) and osteoclasts (Ravesloot et al, 1989), to our knowledge there has been only a few limited studies in growth plate chondrocytes (Grandolfo et al., 1990; Walsh et al., 1992; Vittur et al., 1994).

Until now, all the studies on K^+ channel of chondrocytes were in cell culture model. It was demonstrated that culture time and/or conditions may modify K^+ channels or induce the expression of a new type of channel (Grandolfo et al., 1990). In day three culture, chondrocytes from resting cartilage of pig scapulas, the cells were flat and isolated; they showed an irregular dendritic-like shape, and little or no metachromasia was present. When maintained in an isosmotic (130 mM NaCl, 3 mM

KCl) solution, the chondrocytes adopted a roundish shape. As it was difficult to obtain a high quality and stable gigaohm seal from flat adherent chondrocytes. It was almost impossible to obtain good and stable excised patches due to some not yet-known characteristics of the cultured chondrocyte membrane. As a consequence, cell-attached configuration was used. Voltage dependent K^+ channels with conductance of 168 pS in symmetric potassium detected. In confluent cultures, the cells present a typical polygonal shape. They actively synthesize and secrete proteoglycans, and respond well to parathyroid hormone. The conductance of detected K+ channels changed to 252 pS. Similar phenomena in morphological change during cell culture were observed in pig costal growth plate chondrocytes studied in our laboratory (Referred to Chapter Two). Therefore, in order to avoid differentiation or de-differentiation occurring in culturing condition, it is necessary to establish new model for further characterizing the properties of various types of ion channel in chondrocytes. In the present study, we have successfully developed a new preparation of partially digested growth plate which provides cells of identifiable stages of differentiation and which are suitable for patch-clamp recordings, as evidenced by the dramatic increase in success rate of tight-seal formation from virtually 0 up to 40%.

Basically, very little is known on the electrophysiological properties of chondrocytes. Using conventional intracellular techniques, it was shown (Edelman, 1985) that during differentiation, the resting membrane potential in chondrocytes of the epiphysial plate changes, along with the potassium intracellular activity. However, to our knowledge, very little information is available on the ionic mechanism involved in the regulation of the membrane properties. Grandolfo and his co-workers, as have been mentioned above, first investigated the potassium channels using the patch clamp technique in 1990. This has led to the identification of a K^+-selective, voltage-dependent channel in chondrocytes obtained from resting zone cartilage of pig scapulas in cell-attached recording configuration, and single potassium channels were characterized at different stages of culture (Grandolfo et al., 1990). The outward currents were present, with an open probability increasing with

depolarization, and the K^+ channels showing a mean slope conductance of 121 pS in asymmetric and 252 pS in symmetric potassium solution. Tetraethylammonium (TEA) and quinidine blocked the channels.

In the present study, we confirmed the result of Grandolfo et al (1990) in that the major outward current found in chondrocytes is the delayed K^+ rectifier (Ca^{2+}-independent and voltage-dependent). In addition, for the first time we are able to show that this outward current is present not only in the resting chondrocytes but are expressed in the proliferative and hypertrophic chondrocytes. In fact, the latter two types of cells expressed a much higher magnitude of this current.

The presence of another calcium-activated potassium channels in the same chondrocyte model was reported by the same group two year later (Grandolfo et al., 1992). Results obtained with fura-2 on cultured chondrocytes indicate that the cells respond to an elevation of extracellular calcium concentration ($[Ca^{2+}]_o$) from 0.1 to 2 mM increasing the intracellular concentration of the ion ($[Ca^{2+}]_i$) from 117 to 187 nM. This increment may be blocked by 3mM La^{3+}. Patch clamp experiments in cell-attached configuration showed that, when $[Ca^{2+}]_i$ rises, the open probability (Po) of the K^+ channels increases. Increments in both Po and unitary currents of the K^+ channels can be obtained after applying 2.5μM A23187 with 2mM $[Ca^{2+}]_o$. Hence, the results demonstrate that, in chondrocytes, a class of Ca^{2+} -activated K^+ channels is present and their activity is related to an increase of $[Ca^{2+}]_i$. The conductance of these channels is about 110 pS and it allows them to be classified also as "maxi-K^+ channels" for which the open probability and the open time are strictly related to the level of $[Ca^{2+}]_i$.

In our study, we also observed a Ca^{2+}-dependent K^+ current. The potassium selectivity is confirmed by the reversal potential study, which has similar value to that of the delayed rectifier. This current is also more sensitive to TEA, when compared with the delayed rectifier. The dependency of this outward current to extracellular Ca^{2+} is indicated by the effect of removing extracellular Ca^{2+}. What

seems to be novel is that this Ca^{2+}-dependent current is activated much more strongly when the membrane potential is depolarized to more positive than 40mV. If one assumes that the Ca^{2+}-dependency of this current originates from the opening of Ca^{2+}-channel during depolarisation, one would expect the strongest activation to be around 0mV when the voltage-dependent Ca^{2+}-current is highest. A higher depolarisation would only decrease the driving force for Ca^{2+}-influx. Thus, one is forced to discard this simple model of activation of the Ca^{2+}-dependent outward current. In fact, in the present study, no voltage-dependent inward current has been observed. In this respect, this finding is consistent with that of Grandolfo et al. (1990 & 1992) that the rise in intracellular Ca^{2+}, which activates K^+-current, is not a direct result of voltage-dependent Ca^{2+}-influx. Obviously, further experiments are needed to clarify these issues.

Although the physiological and regulatory roles of the two K^+ channels in growth plate chondrocytes are still not clear, they are likely to be related to chondrocyte maturation. In other non-excitable cells, there is evidence that voltage-gated K^+ channels may play a part in mitogenesis. In the lymphocytes, Cahalan and co-workers (DeCoursey et al., 1984) reported a shift in the conductance vs. voltage curve such that more K^+ channels are open at potentials negative to -20mV when the cells were treated with specific lectin mitogens, such as PHA and ConA, all of which stimulate these cells to proliferate. For the longer-term stimulation with mitogens on cell culture, increase in DNA synthesis as well as K^+ current per cell were demonstrated. Moreover, modulation of potassium conductance is accompanied by altered proliferation. Agents that block the K^+ channel, such as TEA, 4AP, quinine and verapamil inhibit stimulated DNA synthesis. In the present study, the total K^+ current in resting chondrocytes is much lower than those of proliferative and hypertrophic chondrocytes. It seems that K^+ channels are more important in the differentiated chondrocytes, especially for the proliferative chondrocytes which process the highest K^+ current density. It may indicate that K^+ channel is essential for the mitogensis of growth plate chondrocytes.

On the other hand, the mean cell diameter of hypertrophic chondrocytes is about 20 μm which is significantly larger than those of resting and proliferative chondrocytes with 12 and 13 μm respectively. The highest total K^+ current in hypertrophic chondrocytes may indicate that K^+ channel is also critical in cell volume regulation. Some data has been reported on lymphocytes that human T cells regulate cell volume through independent conductive K and Cl pathways. All agents that block these pathways, including those shown to block the K^+ channel in the patch-clamp experiments, prevent regulatory volume decrease (RVD) (Grinstein et al, 1984). Moreover, Deutsch (1990) demonstrated that quinine, a K^+ channel blocker, inhibits this RVD process in a mouse helper T-cell line, L2. Gramicidin, a monovalent cation ionophore, however, accelerates RVD in the presence of an impermeant extracellular cation. Agents that block the limiting permeability pathway block RVD, while those that enhance the limiting permeability, accelerate RVD. Then it is believed that cell swelling activates a chloride conductance, which drives the membrane potential toward the chloride equilibrium potential. The cell is thus depolarized to the voltage-threshold for the K^+ channel, which then opens. Both pathways allow salt and water to exit the cell, thereby restoring isotonic cell volume. It is tempting to hypothesize that similar processes may take place in the chondrocytes in the hypertrophic zones. However, such K^+ channel regulated volume decrease seems to be contradictory to the co-existence of large cell volume and high total K^+ current in hypertrophic chondrocytes. Moreover, it is important to note that the K^+ current per unit plasma membrane area in hypertrophic chondrocytes remain unchanged when compared with the resting chondrocytes. The enlargement of hypertrophic chondrocyte may then represent normal cell growth rather than regulated volume increase / swelling.

Our finding that a relatively higher proportion of the delayed rectifier current is expressed in the proliferative and hypertrophic cells may also indicate that this type of channel play a more active role during the process of differentiation.

Moreover, the high K^+ current detected in the proliferative and hypertrophic chondrocytes may be related to the secretion of Ca^{2+} rich matrix vesicles. Such

specific matrix vesicles production is a well established unique characteristic of proliferative hypertrophic growth plate chondrocytes and has been confirmed by our confocal microscopic study *(Refer to Chapter Four).*

In conclusion, we have developed a new *in situ* model suitable to study the membrane ionic mechanisms of chondrocytes from different differentiation stages. The fact that at least two types of K^+-channels exist on the chondrocyte membrane is consistent with previous findings. We further find out that during differentiation, more outward K^+-currents are expressed which may play critical roles in maintaining a high concentration of extracellular K^+, in the process of mitogensis, cell volume regulation or secretion of Ca^{2+} rich matrix vesicles.

Chapter Six

Effects of Quinolones

on

Growth Plate Chondrocytes

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6.1 AIMS OF STUDY

Since the development of nalidixic acid, quinolone antimicrobials have been used therapeutically for almost 25 years, primarily for treating urinary tract infections. Recently, the clinical usefulness of new quinolones including norfloxacin, ofloxacin and ciproxin have been expanding because of their remarkable antibacterial activities and favorable pharmacokinetics (Neumann, 1988). The quinolones, however, have the common property of inducing cartilage damage in juvenile animals such as vesicle formation in articular cartilage in rats, rabbits and other laboratory animals. However, up to now, most of the studies on quinolones are *in vivo*. In this study we are going to investigate deeply the *in vitro* effect of quinolones on various subpopulations of chondrocytes in a pig rib model. It is expected that cartilage cells at various differentiation stages have different metabolic systems and respond differently to various quinolones.

6.2 LITERATURE REVIEW

Quinolones and Their Use in Pediatric Treatments

Structure and Function

Quinolone drugs are a group of antibacterial chemicals containing the basic ring structure of nalidixic acid. Their common structures are shown in Figure 1. Quinolones impair bacterial DNA-metabolism by inhibition of the enzyme DNA gyrase which is important for bacterial DNA replication and transcription (Hooper and Wolfson, 1991). The revolution in quinolones began during the 1980s with the introduction of norfloxacin. Earlier compounds such as nalixidxic acid, had little therapeutic use due to their limited antimicrobial activities. Norfloxacin differs fundamentally from the older compounds by the presence of a fluorine atom at position 6, hence the name-fluoroquinolones. While norfloxacin has greater antimicrobial activity than the previous quinolone compounds and a much broader spectrum (Wolfson and Hooper, 1988), its use is mainly for the treatment of urinary tract infections (Sabbaj et al, 1985), gastro-intestinal infections and sexually transmitted diseases (Bogaerts et al., 1987) where the causative organisms are predominantly Gram-negative. This is because of its relatively low activity for Gram-positive pathogens, poor tissue distribution, and poor absorption (Bergan and Thorsteinsson, 1986). Recent modifications have been introduced to the structure of the compound in order to enhance the pharmacokinetics and to provide a wider coverage of antimicrobial activity. The newly developed fluoroquinolones such as ciprofloxacin and ofloxacin exhibit a marked efficacy against a wide spectrum of Gram-negative, Gram-positive and anaerobic bacteria (Van Landuyt et al., 1990). Most fluoroquinolones possess good characteristics of pharmacokinetics such as high bioavailability after oral administration, good tissue distribution and low protein binding (Lode et al., 1990).

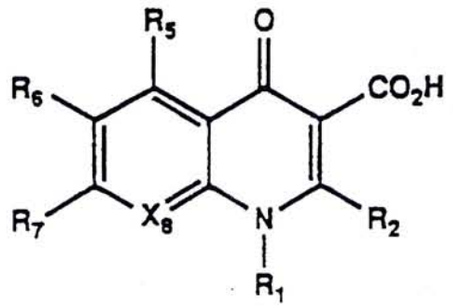

Figure 6.1 The basic double phenol rings of quinolones

Safety

Fluoroquinolones are generally very safe antibiotics which do not cause serious adverse reactions (Wolfson and Hooper, 1991; Maggiolo et al., 1990; Schaad, 1991). The most frequent side-effects are mild gastrointestinal complaints (nausea, dyspepsia, vomiting) and CNS reactions (dizziness, insomnia and headache). Due to potential phototoxcity, caution is necessary when fluoroquinolones, is prescribed to patients who will have intensive exposure to UV light during treatment.

One of the most serious setback of the quinolones, perhaps, is the possible anthralgic effect on growing subject. This has limited their prescription almost exclusively to adult patients. Toxicological studies performed in animals with a wide range of quinolone derivatives showed that virtually all quinolones may cause lesions of the major diarthrodial joints when administered to immature or juvenile animals (Machida et al., 1990). The absolute potency of the various compounds and the sensitivity of different animal species are very variable. The mechanism involved in the development of the arthropathic lesions are unknown. Morphological findings include erosions of the joint cartilage with blister-like degeneration, accompanied by a cell-free non-inflammatory effusion in the joint cavity (Maggiolo et al., 1990). The decomposition of the cartilage matrix can cause an additional irritative reaction to the synovia.

Clinical Data

The prescription of quinolones to pediatric patients (less than 800 cases reported when compared to the estimated treatment of over 1.5 million adult patients) is considered only at difficult situations such as bronchopulmonary exacerbation in patients suffering cystic fibrosis. Arthritis has been reported in a small number of adults and children (Maggiolo et al., 1990). However, the toxicity appeared to be mild and readily reversible after quinolone treatment was terminated. The possible safe use of quinolones in children would provide a greater choice of treatment in the management of pediatric infections.

Cartilage : Growth and Differentiation

Normal Growth and Differentiation

The cartilage is made up of chondrocytes embedded in a matrix which consists largely of water, collagen and proteoglycans. Different types of cartilage are characterized by the composition of the matrix materials. The growth of cartilage is attributable to two processes; interstitial growth, due to mitotic division of the preexisting chondrocytes and appositional growth, due to the differentiation of peripheral perichondrial cells. In both cases newly formed chondrocytes synthesize collagen fibrils and amorphous glycosaminoglycans. Most of the embryonic cartilaginous skeleton is eventually replaced by bone during endochondral ossification in the growth plate cartilage. The endochondral ossification center can be divided into 5 zones: the resting, proliferative, hypertrophic cartilage, calcified cartilage and ossification zones (Junqueira and Carneiro, 1990).

Degenerative Cartilage

Although the pathophysiology of most cartilage degeneration is unknown, there is strong evidence that locally produced factors, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) are important mediators. They cause the sequence of events which lead to the persistence of synovitis and the destruction of cartilage in rheumatoid arthritis and other inflammatory disease of joints (Duff, 1988). IL-1α and -β genes are activated in the synovial cells of patients with rheumatoid arthritis and significant levels of IL-1 are detectable in joint effusions. Chondrocytes also express the mRNA of these two genes and IL-1 activity has been detected in supernatants of bovine articular cartilage. A major part of cartilage invasion is believed to result from the production of arachidonic acid metabolites and proteases by the pannus cell. IL-1 stimulates plasminogen activator and collagenase production. Collagenase is directly capable of degrading cartilage matrix proteins (Gilman et al., 1988). Like IL-1, TNF has similar effects on cartilage destruction in rheumatoid arthritis and inhibits the synthesis of cartilage proteoglycans (Sallatvala, 1986).

In Vitro Study

We hypothesize that cartilage cells at the various differentiation stages have different metabolic systems and respond differently to stimulators and suppressors. The conventional method of obtaining cells from growth plate cartilage is micro-dissection. In this study, we will collect homogeneous resting chondrocytes and heterogeneous growth plate chondrocytes (a matrix of chondrocytes in resting, proliferative and hypertrophic stages of maturation) in a pig rib growth plate model and the effect of quinolone on these cells will be studied.

6.3 METHODS

6.3.1 Isolation of Porcine Chondrocytes

Protocol for isolation of growth plate and resting chondrocytes from porcine costal growth plate cartilage was described in Chapter Two Section 2.3.1.

6.3.2 Chondrocyte Cultures and Quinolone Treatments

Chondrocytes were plated in 24-well culture plates (Corning) at a density of 2×10^5 cell/well in 1 ml DMEM containing 5% FBS, 50 mg/ml ascorbate (Sigma). After the cells have settled for 16 hr in culture, DMEM with above additives plus quinolone (ciproxin, nalidixic acid, ofloxacin or norflorcin) in specific concentrations (0.1, 1, 10 or 100 mg/ml) were used to replace the old medium *(The serum concentration of these quinolones are about 1 mg/ml).* Assays on chondrocytes were performed after the cells have been treated for 24 h in culture. All experiments were done in triplicate.

6.3.3 Cell Viability Determination

After the resting or growth plate chondrocytes have been treated with quinolones for 24 hr, they were released with trypsin (0.1% in PBS, Gibco) for 10 min. The cell viability was determined by trypan blue (Sigma) staining.

6.3.4 Thymidine Incorporation Assay on Cultured Chondrocytes

After the cell have been treated for 24 h in monolayer culture, they were then labeled with 5 μCi/ml [^{3}H]-thymidine (40Ci/mM) (New England Nuclear) in the presence of 5 μM unlabeled thymidine as carrier in DMEM containing 5% FBS for 4 h at 37°C. The medium with excess thymidine was aspirated and the cells were washed

with 1 ml 0.15 M NaCl. The cells were then lysed with 0.25 ml 0.25 M NaOH. After 20 min, attached cells were scraped out with a cell scraper. The lysed cell solution was transferred to a 5-ml plastic tube. Another 0.25 ml NaOH was used to rinse the well of the culture plate and also poured into the same tube. Alkalinity was neutralized by adding 0.5 ml of 0.25 M HCl. One ml of HEPES-Mg-Ca with 2.5 mg/ml bovine serum albumin, which acted as protein carrier, was also added to the tube. Deoxyribonucleic acid (DNA) was precipitated with 0.5 ml of 10 M perchloric acid at 4°C for 20 min. Precipitate was spun at 18,000 x g for 30 min. at 4°C . After aspirating the supernatant, 0.5 ml of 0.25 M NaOH was added to resuspend the pellet. The resuspended solution was transferred to counting vials and 4 ml of scintillation fluid was added. The radioactivity of the acid-insoluble DNA content was counted by a Beckman Liquid Scintillation Spectrometer. Standards of the radiolabeled medium were prepared for direct calculation of the incorporation of radioactive thymidine into DNA.

6.3.5 Sulfate Incorporation Assay on Cultured Chondrocytes

In this assay, cell cultures were exposed to 12 μCi/ml of $^{35}SO_4^{2-}$ sodium salt (DuPont NEN, Boston) in DMEM, which contained 0.814 mM carrier sulfate, for 4 h at 37°C. After incubation, the medium was transferred into a small plastic tube. Cells were lysed by adding 0.25 ml of 0.25 M NaOH to each well and left for 20 min. Cells were then scraped out with a cell scraper and were transferred to the same plastic tube. Another 0.25 ml NaOH was used to rinse the well of the culture plate and transferred to the tube. After adding 0.75 ml of 0.15M NaCl, the solution was dialyzed against phosphate buffered saline, pH 7.4, using 12,000 - 14,000 MW dialysis tubing (Spectrum Medical , Los Angeles) in order to remove unincorporated radiolabeled sulfate. Phosphate buffered saline were changed twice a day. After 3 days, 0.75 ml of the sample inside the dialysis tubing was placed into a scintillation vial and into which 4 ml of scintillation fluid was added. The radioactivity of $^{35}SO_4^{2-}$

incorporated macromolecules was quantified by a Beckman scintillation spectrometer. Data are expressed as nmol of sulfate incorparation per 10^6 cells per hour (nmol/10^6 cells/h)

6.3.6 Alkaline Phosphatase Assay on Cultured Chondrocytes

The enzyme activity of alkaline phosphatase was by colorimetric method with p-nitrophenyl phosphate as the substrate (O'Keefe et al., 1989). Assays were performed directly in the culture wells containing approximately 2 x 10^5 cells/well after the isolated chondrocytes have been treated with quinolones for 24 hr. The culture medium was aspirated from the wells, the cells were then rinsed with 150 mM NaCl solution and the supernatant again aspirated. One ml of reaction buffer containing 0.25 M 2-methyl-2-amino propanol, 1 mM $MgCl_2$, and 2.5 mg/ml p-nitrophenyl phosphate (Sigma) at pH 10.3 was then added to the wells at 37°C to initiate the reaction. After the reaction was stopped by adding 0.5 ml 0.3 M trisodium phosphate, pH 12.3, the absorbance at 410 nm was measured using 0.15 M NaOH as blank. The activity of alkaline phosphatase was calculated from a standard curve prepared by using p-nitrophenol and expressed as μM/10^6 cells/h.

6.4 RESULTS

6.4.1 Cell viability

Cell toxicity effect of various quinolones on resting chondrocytes was demonstrated in this study (Figure 6.2). Ciproxin and nalidixic acid killed about 10% of resting chondrocyte at 0.1 μg/ml while ofloxacin and norflorcin did not show any effect at such concentration. For drug concentration between 1 to 100 μg/ml all type of quinolones tested except ofloxacin exhibited similar effect on cell viability which maintained at about 90% of control. There was a dose dependent cytotoxic effect for ofloxacin at concentration between 0.1 to 10 μg/ml. Ofloxacin seems to be most potent in cell toxicity that it killed about 20% of cells for drug concentration over 10 μg/ml while other quinolones killed about 10% of cells at the same dose.

All quinolones did not affect the cell viability so much on growth plate chondrocytes as showed in Figure 6.3. At drug concentrations less than 1 μg/ml, these 4 different types of quinolones seem not to exhibit any effect on cell viability, especially ofloxacin showed no significant effect even at concentration of 10 μg/ml. The rest of quinolones tested demonstrated a dose dependent killing effect at concentration between 1 to 100 μg/ml.

6.4.2 DNA Synthesis Potential

Figure 6.4 illustrates the result of quinolones on thymidine uptake in resting chondrocytes. At a concentration less than or equal to 1 μg/ml, all quinolones tested, except ofloxacin, had no significant effects on chondrocyte. For the

quinolone concentration between 1 to 100 μg/ml, dose dependent inhibition effect were demonstrated. Nalidixic acid was the most potent inhibitor on thymidine uptake that more than 96% inhibition was observed. On the other hand, ofloxacin showed a bi-phasic effect on thymidine uptake in resting chondrocytes. At concentration between 0 to 1 μg/ml, there was a dose dependent stimulation while a dose dependent inhibition response was obtained at concentration between 1 to 100 μg/ml.

There was a slightly inhibition (not significant) for all the quinolones tested on the thymidine uptake ability in growth plate chondrocytes (Figure 6.5). At very high concentration, 100 μg/ml, significant inhibition was observed and norflorcin was the more potent one.

6.4.3 Proteoglycan Synthesis Potential

The effect of various quinolones on the proteoglycan synthesis potential in resting growth plate chondrocytes are irregular (Figure 6.6). For the ofloxacin as well as norflorcin at 0.1 μg/ml and ciproxin at 1 μg/ml, these three quinolones could stimulate the sulfate incorporation rate ($p < 0.05$). However, at 100 μg/ml, both ciproxin and ofloxacin exhibited suppression effect. Nalidixic acid could not cause any significant variation in sulfate incorporation.

Although the nalidixic acid also had no significant effect on proteoglycan synthesis potential in growth plate chondrocytes, there was a general trend of inhibition for the rest of quinolones tested (Figure 6.7). Norflocin was the potent inhibitor, since 65% of the control sulfate incorporation rate was measured in such a low concentration of 0.1 μg/ml. This specific rate was maintained in the presence of 0.1

to 10 μg/ml of norflocin. When the norflocin concentration increased to 100 μg/ml, more than 80% of proteoglycan synthesis activity was suppressed. Ciproxin and ofloxacin showed similar inhibition effect but with less extent.

6.4.4 Alkaline Phosphatase Activity

The alkaline phosphatase activity in resting chondrocytes was minimal and did not show any response to all the quinolones tested in this study even in the highest concentration (Figure 6.8).

In growth plate chondrocytes, alkaline phosphatase was actively produced. Its production was suppressed by all the quinolones tested in this study. The effective concentration started at 0.1 μg/ml and inhibition was maintained in higher concentration. The maximal inhibition (18%) was observed in 1 μg/ml of ciproxin treatment (Figure 6.9).

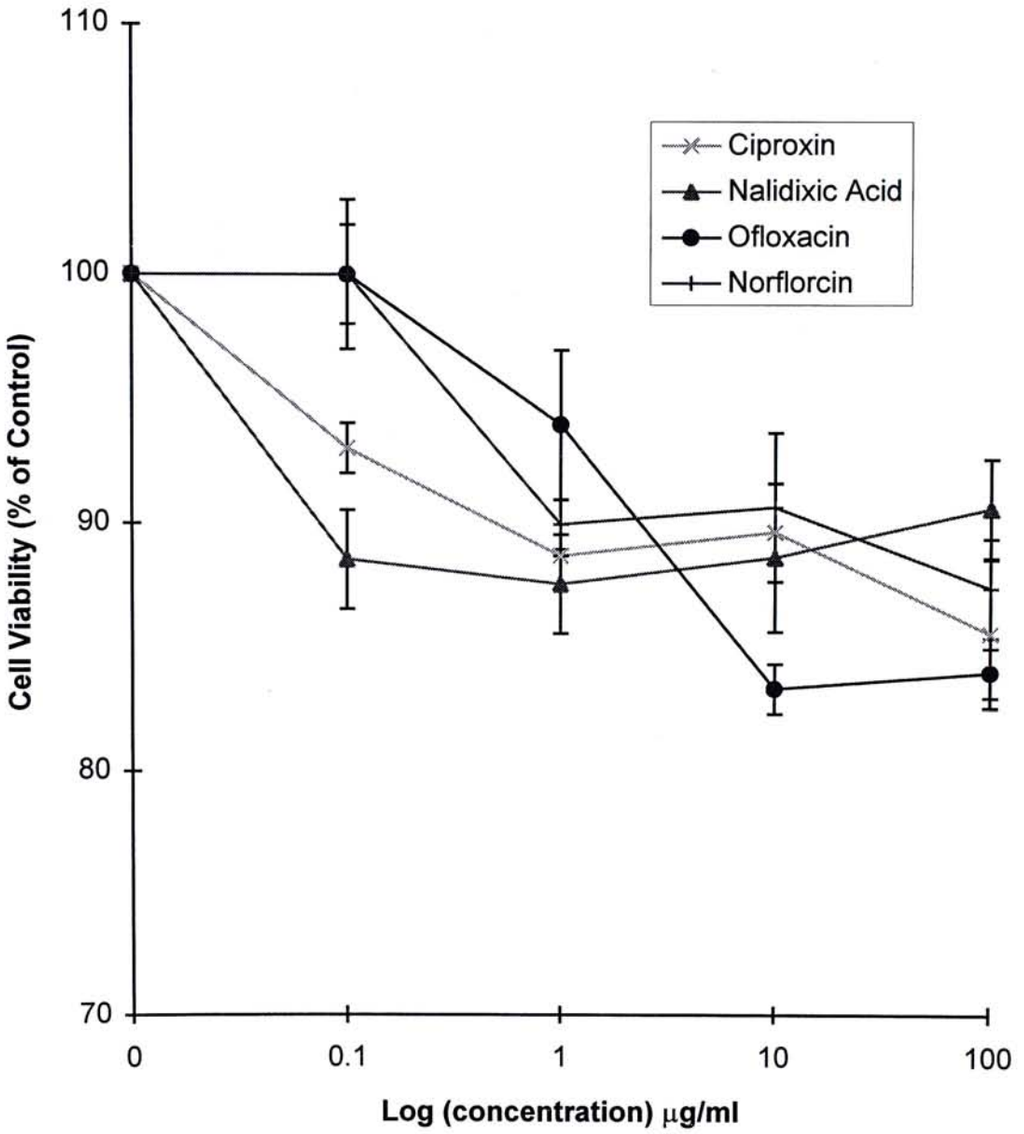

Figure 6.2 Effects of Quinolones on Cell Viability in Porcine Costal Resting Chondrocytes. The cell viabilities are measured after 24 hr treatment and expressed as % of control, 0 mg/ml quinolone concentrations. Data are expressed as mean + S.D. (n=5).

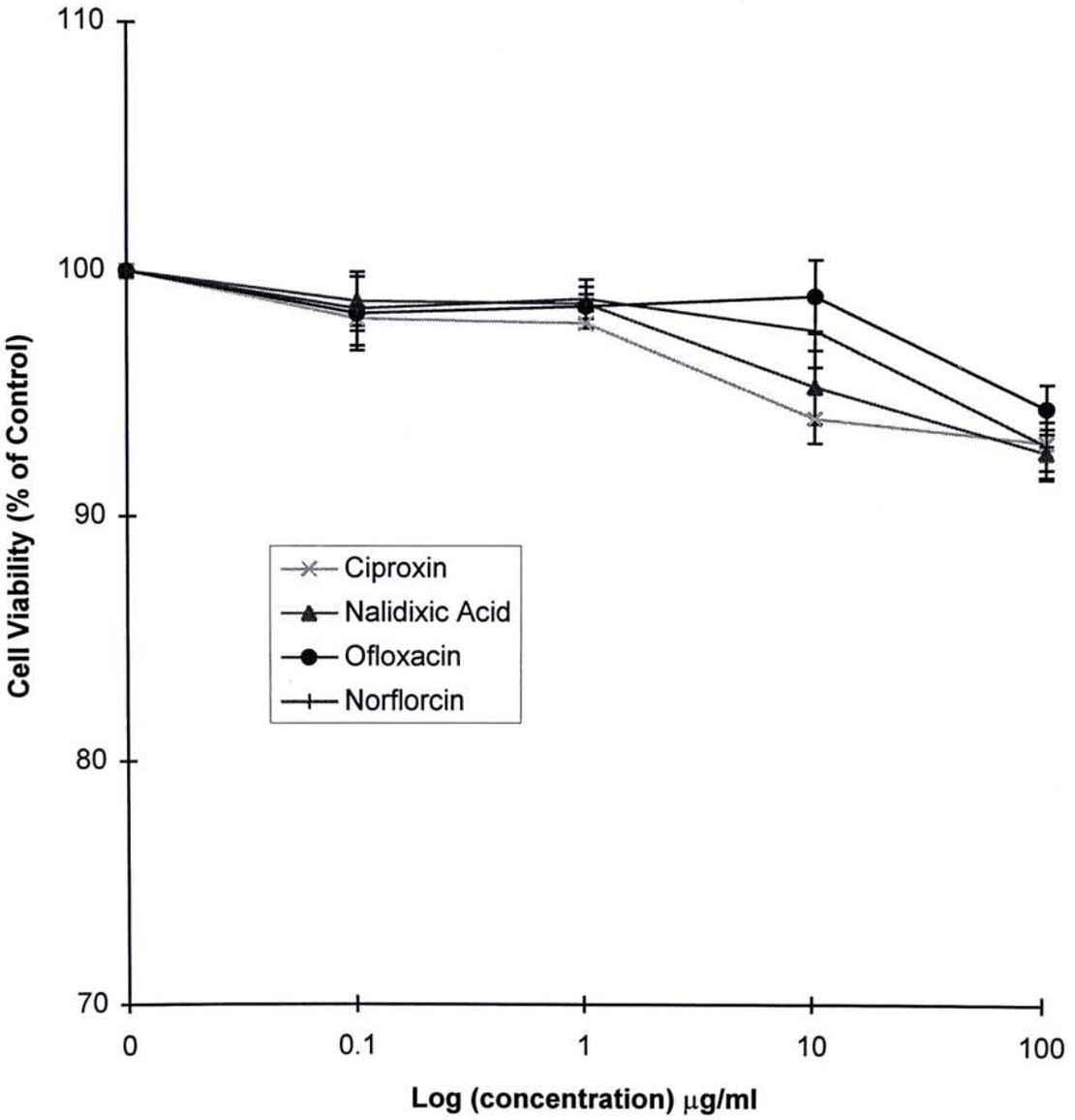

Figure 6.3 Effects of Quinolones on Cell Viability in Porcine Costal Resting Chondrocytes. The cell viabilities are measured after 24 hr treatment and expressed as % of control, 0mg/ml quinolone concentrations. Data are expressed as mean + S.D. (n=5).

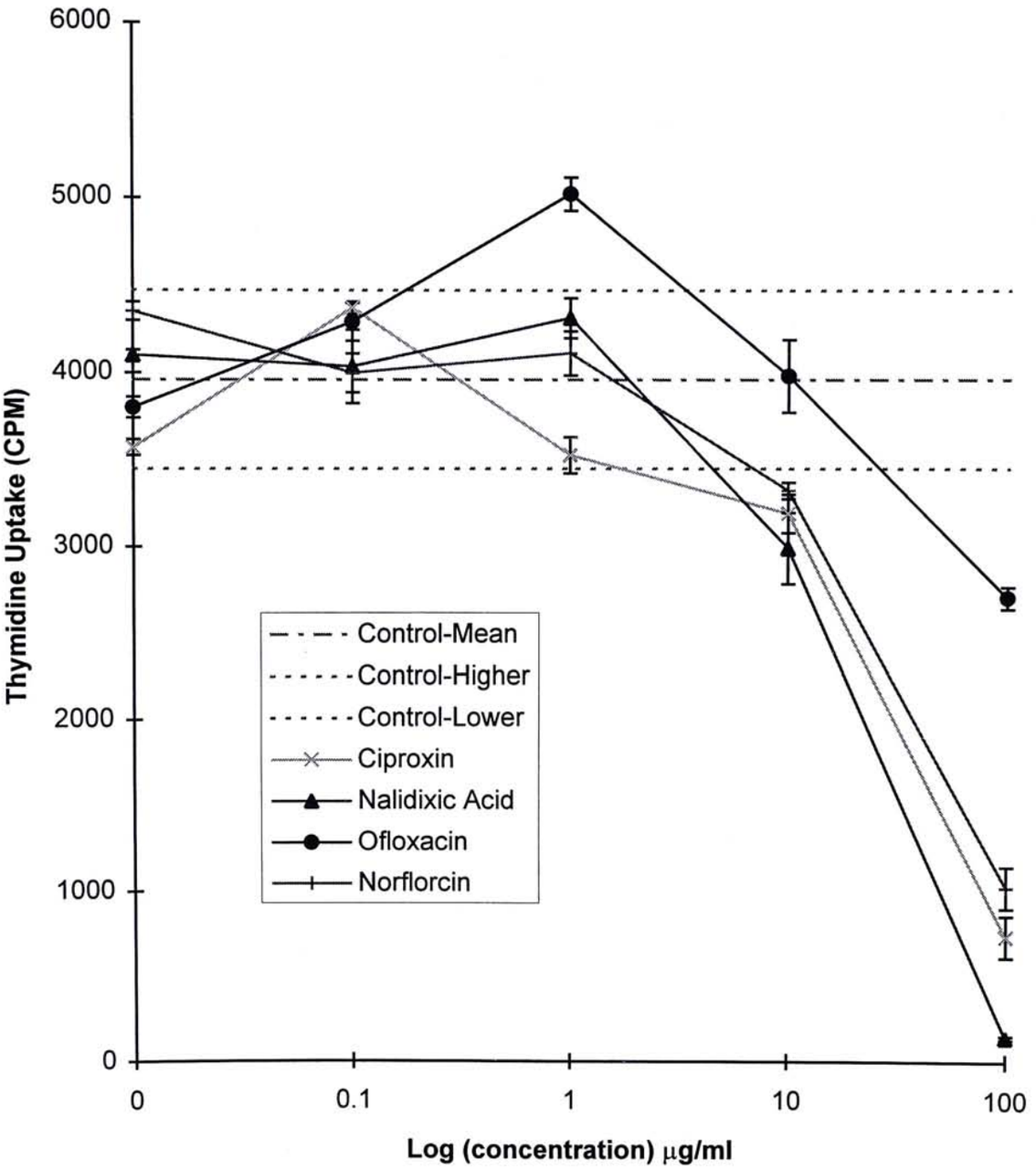

Figure 6.4 Effects of Quinolones on Thymidine Uptake in Porcine Costal Resting Chondrocytes. The incorporation rates of radiolabeled thymidine were determined after 24 hr of treatment. Data are expressed as mean ± S.D. (n=5).

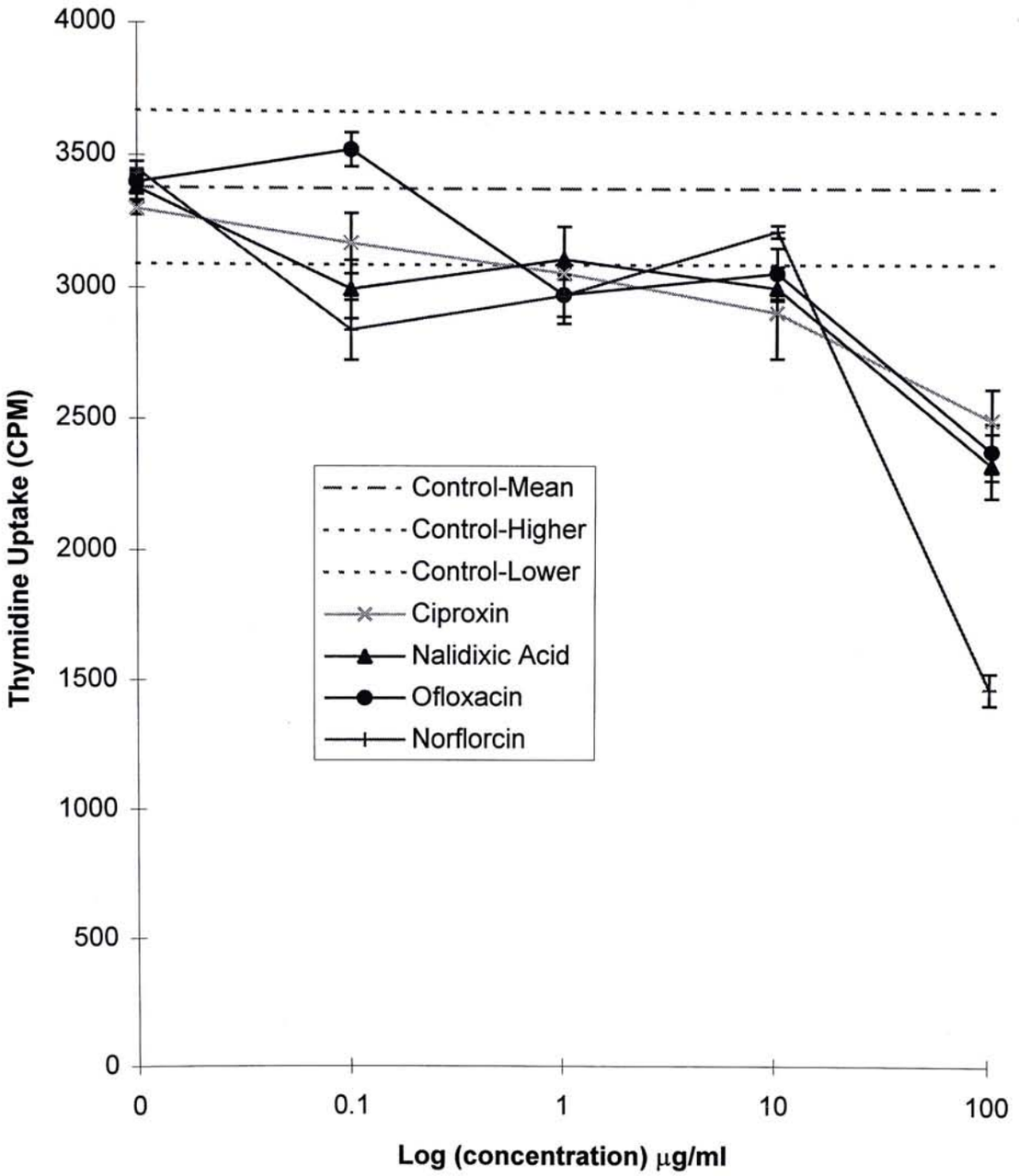

Figure 6.5 Effects of Quinolones on Thymidine Uptake in Porcine Costal Growth Plate Chondrocytes. The incorporation rates of radiolabeled thymidine were determined after 24 hr of treatment. Data are expressed as mean ± S.D. (n=5).

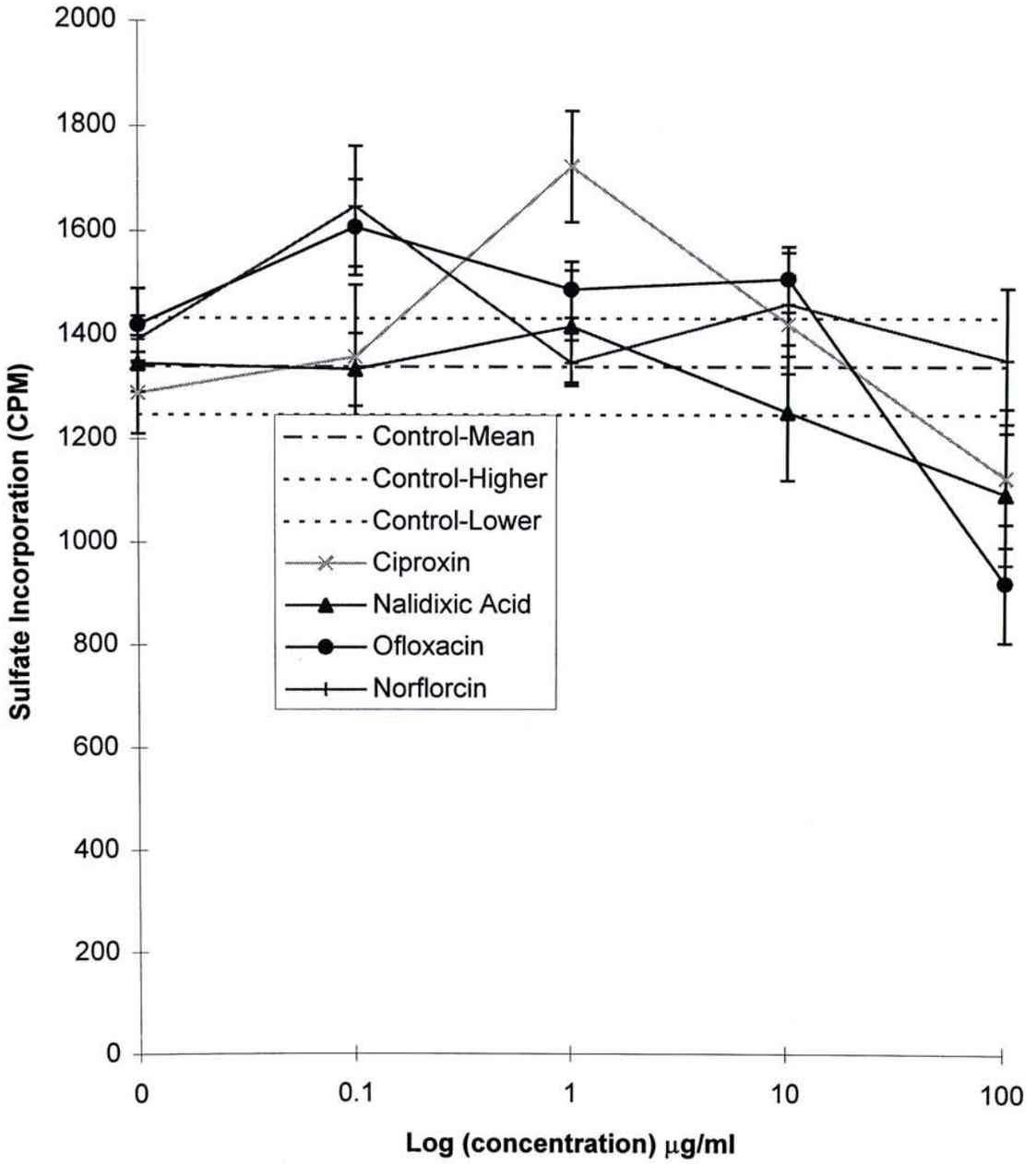

Figure 6.6 Effects of Quinolones on Sulfate Incorporation in Porcine Costal Resting Chondrocytes. The incorporation rates of radiolabeled sulphate were determined after 24 hr of treatment. Data are expressed as mean ± S.D. (n=5).

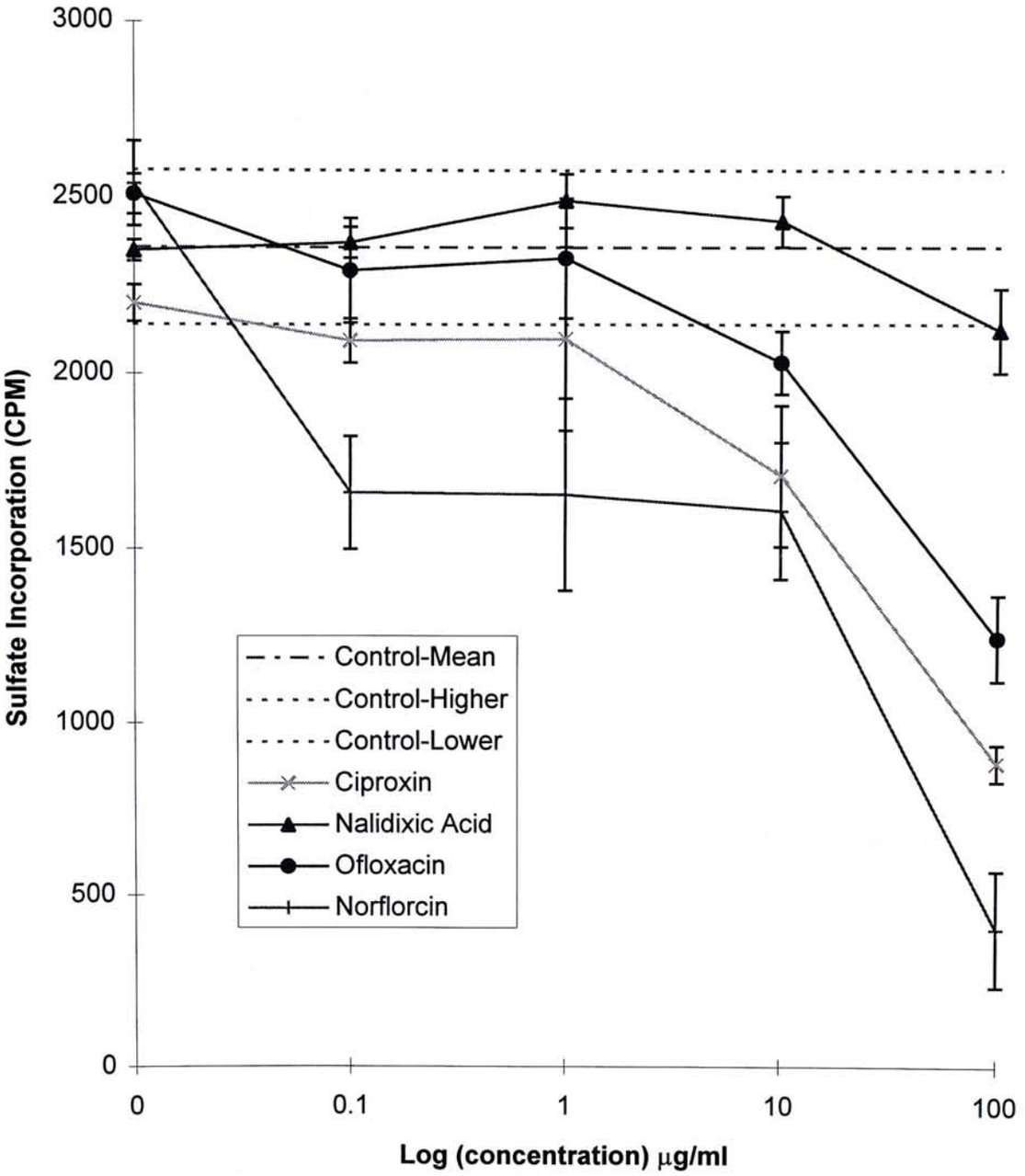

Figure 6.7 Effects of Quinolones on Sulfate Incorporation in Porcine Costal Growth Plate Chondrocytes. The incorporation rates of radiolabeled sulphate were determined after 24 hr of treatment. Data are expressed as mean ± S.D. (n=5).

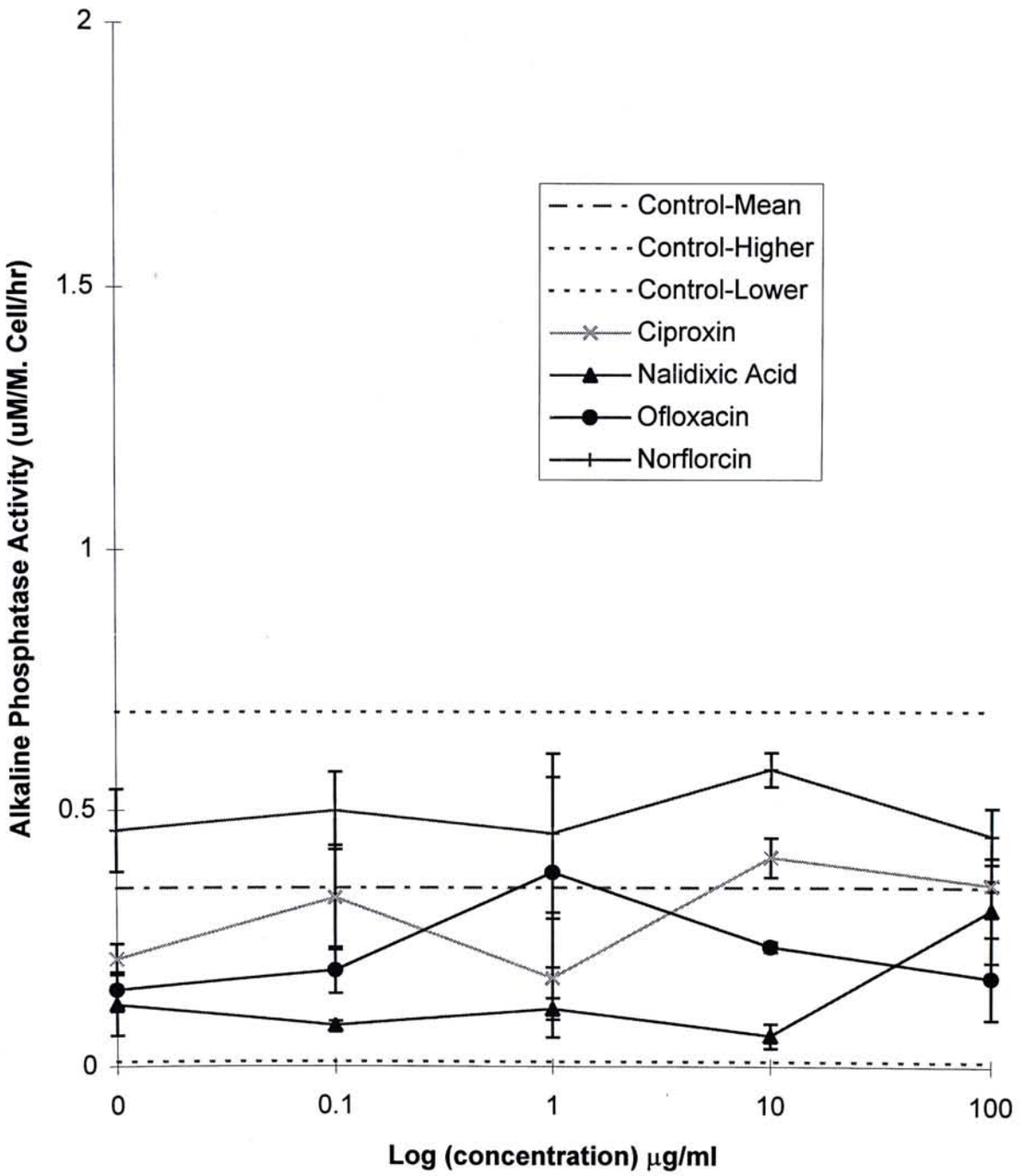

Figure 6.8 Effects of Quinolones on Alkaline Phosphatase Activity in Porcine Costal Resting Chondrocytes. The enzyme activities were measured after 24 hr of treatment. Data are expressed as mean ± S.D. (n=5).

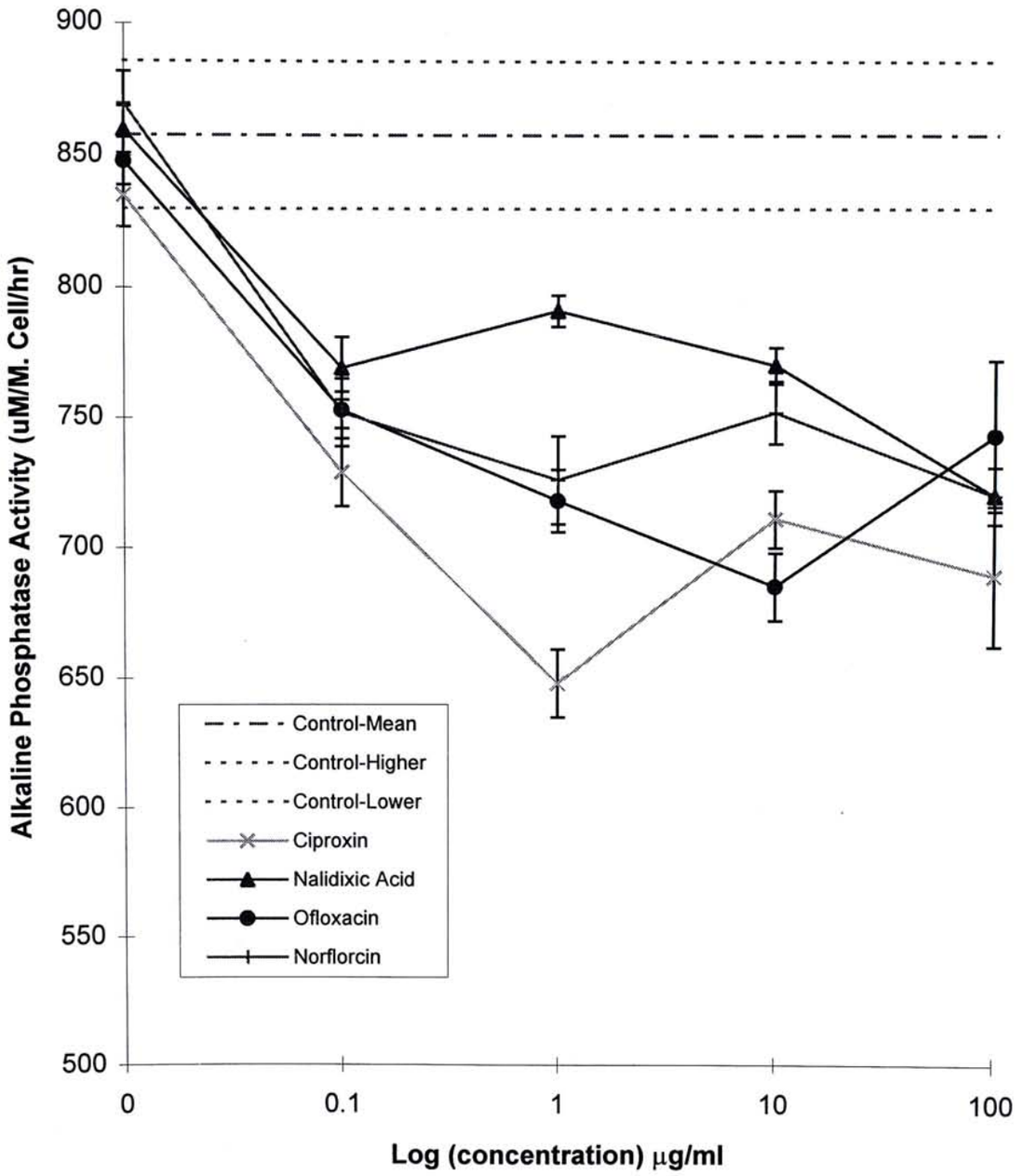

Figure 6.9 Effects of Quinolones on Alkaline Phosphatase Activity in Porcine Costal Resting Chondrocytes. The enzyme activities were measured after 24 hr of treatment. Data are expressed as mean ± S.D. (n=5).

6.5 DISCUSSION

Following the discovery of nalidixic acid in 1962, numerous structural modifications have been made to the quinolone nucleus to increase antimicrobial activity and improve pharmacokinetic performance. A major advance occurred during the 1980s with the discovery that a fluorine at position 6 conferred broad and potent antimicrobial activity, e.g. norfloxacin. However, the derivatives still have relatively less activity for Gram-positive and anaerobic organisms than Gram-negative bacteria. Subsequent developments produced quinolones with further improvements, predominantly in either solubility (e.g. ofloxacin) antimicrobial activity (e.g. ciprofloxacin) or prolonged serum half-life (Percival, 1991). The side effect on cartilage damage of nalidixic acid, norflorcin, ofloxacin and ciprofloxacin, four quinolones of different stage of drug development were not fully understood.

From the result of this study, quinolones affect the cell viability of resting chondrocytes much seriously than the growth plate chondrocytes, as evidence by our findings that the lowest cell viabilities obtained after treatment in resting and growth plate cells are 83% and 93% respectively. Moreover, for the drug concentration of 1 μg/ml (serum concentration), all quinolones tested showed significant inhibition on resting chondrocytes viability while only minimal effect on growth plate chondrocytes were detected. Chondrocytes released from these two different cartilage regions showed different response to ofloxacin. Among the four quinolones tested in this study, ofloxacin appears to be the most potent cytotoxic drug for resting chondrocytes but is the least potent one for growth plate.

Several previous studies have confirmed the peculiar susceptibility of growing animals to the chondrotoxicity effect, whereas maturity seems to confer resistance (Gough, 1992; Ribard, 1991). Histologic lesions of articular cartilage appear very

quickly after treatment initiation and begin in the intermediate cartilage zone. Ultra-structural studies have shown early changes with chondrocytes, such as in swelling of mitochondria and the appearance of intracytoplasmic vesicles (Burkhardt, 1992; Stahlmann 1990). Hayem and his co-workers (1994) demonstrated a significant increase in the respiratory burst in all ofloxacin and pefloxacin treated young rabbits. No significant increase of respiratory burst was noted in older treated rabbits. Fluoroquinolone arthrotoxicity seems to be involved in its early phase of the respiratory burst of immature articular chondrocytes.

Thymidine is one of the essential building block of DNA, the thymidine incorporation rate reflects the DNA synthesis activity and in term the cell proliferating potential. Although at or less than 1 μg/ml, no significant effect of quinolones on chondrocytes was observed. All quinolones (except ofloxacin) tested in this study showed a general inhibition on thymidine uptake in both resting and growth plate chondrocytes at higher concentrations. The effect seems to be more potent in resting chondrocytes than growth plate chondrocytes. It was because at the concentration of 100 μg/ml, the maximum inhibition on thymidine uptake was 94% in resting chondrocytes while it was only 57% in growth plate chondrocytes. Although such high concentration of quinolones seems to be not physiological, for rabbits receiving a dose of 200 mg/kg quinolone, the maximum serum and articular cartilage concentration of norfloxacin were 51.2 μg/ml and 46.3 μg/g respectively and those concentrations on nalidixic acid used in the treatments were even higher (Machida et al., 1990). Recently, Hayem and his co-workers (1994) demonstrated the local concentration of pefloxacin, a new type of fluroquinolone, in cartilage is 91.54 μg/g. Thus, the inhibition effect of quinolones on cell division in high concentrations could be occurred physiologically.

Suppression effect of [^{3}H] thymidine uptake by ofloxacin in rat articular cartilage cell was also reported (Kato and Onoder, 1988). In this study, ofloxacin however

demonstrated a bi-phasic effect on thymidine uptake in resting chondrocytes but not growth plate chondrocytes. At concentration between 0 to 1 μg/ml, there was a dose dependent stimulation while a dose dependent inhibition response was obtained at concentration between 1 to 100 μg/ml. Although such uncommon bi-phasic phenomenon cannot be explained, it is the factual finding after a lot of repetition of experiments.

From the result of this study (Figure 6.6 and 6.7), the proteoglycan synthesis activities in growth plate chondrocytes are almost twice of those in resting chondrocytes. It indicates that growth plate chondrocytes are active in matrix production while resting chondrocytes are really in a quiescent stage.

Proteoglycan and collagen are the major components of cartilage matrix which are produced by chondrocytes. Keratin sulfate and chondronitine sulfate are the most predominant proteoglycan in cartilage. Then the sulfate incorporation rate reflect the matrix production activity. For proteoglycan synthesis activities, although nalidixic acid showed no effect on both resting and growth plate chondrocytes, the effect of other quinolones are confusing in resting when compared with growth plate chondrocytes. In growth plate chondrocytes, all the other quinolones perform a suppression on sulfate incorporation. Norflorcin is the most potent inhibitor. Similar inhibition effect of ofloxacin on sulfate uptake in rat articular cartilage was reported by Kato and Onodera in 1988. On the other hand, nalidixic acid demonstrated no effect while ciproxin and ofloxacin showed bi-phasic effect on sulfate incorporation in resting chondrocytes. Stimulatory response was even detected with norflorcin in 0.1 μg/ml, which showed the opposite response in growth plate chondrocytes. Ultrastructural disruption of extracellular matrix in dog cartilage after oral difoxacin administration was observed in the pericellular matrix of necrotic chondrocytes, indicating that this change was secondary to the changes in chondrocytes. Fissures

within cartilage apparently resulted from the loss of the normal association of proteoglycans with collagen fibrils (Burkhardt et al., 1992).

Alkaline phosphatase is a well established differentiation marker for growth plate chondrocyte. Although the physiological function of this enzyme is still not known, evidence show that it is involved in the mineralization process. Therefore, its activity in resting chondrocytes is minimal when compared with growth plate chondrocytes. It is clear that it will not be influenced by quinolones. However, all quinolones tested in this study can suppress the alkaline phosphatase activity of growth plate chondrocytes. It indicates that quinolones will inhibit chondrocyte differentiation and in term hinder the development of growth plate and then suppress the bone growth.

The pathological effects of quinolones on resting zone and growing zone cartilage are different. In resting chondrocytes, their primary effect are killing the cells directly. In the growth plate cartilage which contain resting, proliferative and hypertrophic chondrocytes, the major pathology of quinolones is preventing chondrocyte differentiation. These observations provide further evidence for the hypothesis that chondrocyte at various maturation stages have different phenotypic characteristics and respond differently among modulators.

Chapter Seven

SUMMARY AND CONCLUSION

The process of endochondral ossification occurring in growth plate cartilage is critical in skeletal development, bone growth and fracture healing. During this process, growth plate chondrocytes differentiate from resting stage through proliferative stage to terminal hypertrophic stage in a highly organized manner. In order to understand better the regulatory mechanism of endochondral ossification, which in term would promote bone growth and fracture healing, it is important to investigate the biochemical and physiological characteristics of chondrocytes in different maturation stages.

Due to the technical difficulties in obtaining pure chondrocyte in different stages of maturation during endochondral ossification, most of the previous investigations on growth plate cells used a mixed population of chondrocytes. We hypothesize that cartilage cells at different differentiation states are different in their metabolism and responses to various hormones and stimulators. Using *in vitro* and *in situ* porcine costal growth plate models established in this study, we successfully identified and separated various differentiative chondrocyte subpopulations. In addition, their phenotypic characteristics, including basic biochemistry, glycoconjugate expression, calcium metabolism, electrophysiology and drug response have been investigated.

In order to isolate relatively pure growth plate chondrocyte subpopulations, countercurrent centrifugal elutriation have been successfully applied to separate, on the basis of size and density, growth plate chondrocytes in pig (Chapter Two). After elutriation, eighteen consecutive fractions were obtained. The mean cellular volumes increased progressively in each of successive fractions and that increase was accompanied with specific phenotypic changes such as differences in DNA synthesis (proliferative potential), proteoglycan (cartilage matrix) synthesis and activities of alkaline phosphatase (differentiation marker). Three distinct maturational growth plate subpopulations each with their own unique biochemical characteristics were identified among the elutriated fractions. The resting chondrocytes were small and quiescent. The hypertrophic chondrocytes were large and metabolically active in alkaline phosphatase as well as proteoglycan productions. The proliferative

population represents the cells with high DNA synthesis potential and its size range lies between the resting and hypertrophic cells.

Glycosylation of proteins is one of the most important post-translational modifications. It determines many of the final structural and functional properties of glycoproteins and glycolipid. It is hypothesized that growth plate chondrocytes and the surrounding matrix at various maturation stage are different in their glycolipid and glycoprotein. Differential expressions of glycoconjugates during endochondral ossification have been investigated (Chapter Three). In this study, the glycoconjugates of intracellular component and extracellular matrix of growth plate cartilage *in situ* were analyzed by lectin histochemistry.

The results showed that glycoconjugates rich in N-acetylgalactosamine and galactose residues appeared to be prominent in the hypertrophic zone matrix as shown by their positive stainings with RCA-I, RCA-II, PNA, DBA, Jacalin and WFA. These N-acetylgalactosamine and galactose containing glycoconjugates in the matrix became increasingly abundant from resting zone to the hypertrophic zone. Moreover, β-galactose was commonly expressed in growth plate chondrocytes, since chondrocytes in all zones were positively labeled with PNA, RCA-I and RCA-II. Previous studies have shown that the distribution of the RCA-I-binding glycoconjugates in hypertrophic chondrocytes was identical to the distribution of fibronectin in the rabbit (Weiss and Reddi, 1981). Then Font and Aubrey (1983) further proved that fibronectin was an RCA-I binding glycoprotein. Therefore, the RCA-I binding glycoconjugates demonstrated in the growth plate cartilage in this study may represent the fibronectin.

S-Con A, a α-mannose binding lectin, bound to the membranes of proliferative and hypertrophic chondrocytes. The cytoplasm of chondrocytes at all zones were also stained. These findings suggest that α-mannose may be essential to growth plate chondrocytes. This observation is similar to that of Nagano (1992) on the physeal cartilage plate of rabbit tibia. The Con A binding to intracellular glycogen has been

reported in the keratinocytes of the skin (Hyun et al., 1984). Pollesello et al. (1991) demonstrated by ^{31}P-NMR that resting and hypertrophic chondrocytes of pig scapulas growth plate depend on glycolysis for their energy production. Thus, the intracellular binding with S-Con A in the chondrocytes probably represents stored glycogen.

On the other hand, glycoconjugates with sialic acid or N-acetylglucosamine are not expressed in the growth plate cartilage since almost all sialic acid and GluNAc specific lectin showed negative binding.

The expression of some glycoconjugates from the differentiating chondrocytes appeared to be stage-dependent during the process of endochondral ossification. For example, S-Con A binding glycoconjugates were expressed during the maturation process of chondrocytes. Moreover, the chondrocytes in the resting zone were positive to VVA, while such staining disappeared in proliferative and hypertrophic zones.

The results of the lectin histochemistry showed that the differentiating chondrocytes in the porcine physeal growth plate express characteristic lectin binding patterns (Summarized in Table 3.4 of Chapter Three). In the physeal growth plate, WGA bound specifically to the resting chondrocytes, while DBA and PHA-E bound to the proliferative and hypertrophic zone chondrocytes. These lectins can be used as the differentiation markers for the growth plate chondrocytes in future studies.

There should be technical difficulties in transforming some histochemical findings into an *in vitro* situation. However, besides countercurrent centrifugal elutriation, separation of chondrocyte subpopulations is possible by lectin affinity chromatography or flow cytometry with fluorescein-labeled lectin probes based on their differential lectin binding to chondrocytes. For example, the chondrocytes released from costal growth plate by enzyme digestion can be stained with FITC-labeled WGA and Cye 5-labeled DBA. The fluorescein-labeled chondrocyte mixture

can be separated by flow cytometery. Then three chondrocyte subpopulations, namely resting (double labeled), proliferative (non-labeled) and hypertrophic (DBA labeled) could be obtained. The separated chondrocyte subpopulations could be used for further biochemical and molecular characterization.

In the growth plate chondrocytes, the content of intracellular free calcium appears to increase during the processes of cellular maturation (from resting stage, through proliferative stage to hypertrophic stage) and matrix mineralization (Iannotti and Brighton, 1989). The physiologic significance of the accumulation of large amounts of intracellular free calcium $[Ca^{2+}]_i$, in the growth plate chondrocytes has not yet been determined but it has been postulated to play a role in matrix mineralization and regulation of cellular metabolism. Since attachment of cartilage matrix is critical for maintaining the physiological behavior of calcium metabolism in chondrocytes, a new model for *in situ* monitoring of intracellular and extracellular Ca^{2+} with laser scanning confocal microscopy from each zone of the porcine growth plate was established (Chapter Four).

From the results of this study, there appears a general trend for the intracellular free calcium to concentrate in the nuclei of the resting chondrocytes through the proliferative chondrocytes to the sub-plasma membrane region in the hypertrophic chondrocytes. This indicates that in the hypertrophic chondrocytes and proliferative chondrocytes of maturation zone, free calcium is mainly trapped inside the Golgi apparatus and endoplasmic reticulumn for cellular secretion through matrix vesicles. This finding has been supported by the evidence that free calcium arcs and islands were detected in the hypertrophic chondrocytes and proliferative chondrocytes respectively.

In physeal chondrocytes, TGF-β was shown to stimulate proteoglycan synthesis while having no effect on collagen or DNA synthesis (O'Keefe et al., 1988). TGF-β is also a potent promoter of the differentiation of mesenchymal cells into chondrocytes (Seyedin et al., 1985). The effects of TGF-β on growth plate

chondrocytes in various stages of maturation have been investigated with the *in situ* model established in this study. Since most of the previous *in vitro* studies were performed on growth plate chondrocytes which contained mixtures of chondrocytes at different maturation stages, the differential responses of individual chondrocytes subpopulations have not been fully investigated. The results of this study demonstrated that there are differential responses on intracellular calcium in growth plate chondrocytes among various zones of growth plate to TGF-β_1. In resting chondrocytes, all cells showed a significant increase in calcium level after adding TGF-β_1. In proliferative chondrocytes, some cells were responsive to TGF-β_1 while others were not. For the responsive cells, the stimulation of intracellular calcium was much more mild when compared with resting chondrocytes. There was no response on intracellular calcium to TGF-β_1 in the hypertrophic chondrocytes.

Analysis of the inhibitory effects of TGF-β on the cell cycle of a variety of different cell types demonstrates that it results in cell arrest in the middle to late G_1 phase, delaying entry into the S phase (Heimark et al., 1986; Chambard and Pouyssegur, 1987). We therefore postulate that the localization of the effectiveness of TGF-β_1 in all the resting and some of the proliferative growth plate chondrocytes may indicate that the removal of the TGF-β_1 responsiveness is essential for triggering of cell division from the resting chondrocytes and further differentiation of the proliferative chondrocytes. Whether this is achieved by the disappearance of TGF-β_1 receptors in the proliferative and hypertrophic chondrocytes requires further studies.

In this study, triggering of intracellular Ca^{2+} increase by the binding of TGF-α to the growth plate chondrocytes for all of the three maturation stages has been demonstrated. The binding of TGF-α to receptor leads to activation of the receptor's tyrosine kinase activity and resulting in increased IP_3 production. This is then followed by an increase in intracellular Ca^{2+} as a result of release from intracellular stores in response to IP_3 and activation of protein kinase C.

It is a well established fact that parathyroid hormone (PTH) regulate the calcium level in plasma and bone. However, in the porcine costal growth plate chondrocyte model established in this study, the effect of PTH on chondrocytes does not seem to involve Ca^{2+} as a secondary messenger. Similar findings that calcium transients are not involved in the transduction of PTH effects on avian growth plate chondrocytes have been reported recently (Zuscick et al., 1995).

Very little has been known about the electrophysiological properties of chondrocytes, although the transmission of signals mediated by ion channels within and between cells is critical for cell differentiation. Grandolfo and his co-workers first investigated the potassium channels using patch clamp technique in 1990. Among the limited investigations on chondrocyte ion channels, all the studies involved cell culture models. It was well known that differentiation and de-differentiation of chondrocytes under culture condition is unavoidable. Moreover, since the CCE fractions are only highly enriched with certain chondrocyte subpopulations, certain percentage of cross contamination by other subpopulations cannot be eliminated. This would create problems for some individual cell physiology assessments. To avoid this problem, an *in situ* model of partially digested growth plate slices for further characterization of different types of ion channel in various differentiation stages of growth plate chondrocytes has been established (Chapter Five). Two types of K^+-channels have been found on the chondrocyte membranes and subsequently identified.

In this study, we demonstrated that the major outward current found in chondrocytes is the delayed K^+ rectifier which is consistent with the findings of Grandolfo et al (1990). In addition, for the first time we were able to show that this outward current is present not only in the resting cells but were also expressed in the proliferative and hypertrophic cells. In fact, the latter two types of cells expressed a much higher magnitude of current. This may indicate that such type of channel plays an active role during the process of differentiation. The presence of another calcium-dependent

potassium current has been identified in chondrocytes. This current is found to be more sensitive to TEA when compared with the delayed rectifier.

Moreover, a high K^+ current is detected in the proliferative and hypertrophic chondrocytes which may be related to the secretion of Ca^{2+} rich matrix vesicles. Such specific matrix vesicle production is a well established unique characteristic of proliferative and hypertrophic growth plate chondrocytes which has been confirmed by our confocal microscopic study (Chapter Four). The findings that during differentiation, more outward K^+-currents are expressed may imply such channels play a critical role in maintaining a high concentration of extracellular K^+, in the process of mitogensis, cell volume regulation or secretion of Ca^{2+} rich matrix vesicles.

In order to further demonstrate that chondrocytes at various stages of differentiation possess their own phenotypic characteristics and respond distinctly with different hormones and modulators, the differential effects of quinolones, a clinically useful broad spectrum antibiotics, which is known to have the side effect of inducing cartilage damage, in different chondrocyte subpopulations was studied in Chapter Six.

From the results of this study, it appears that quinolones such as ciproxin, ofloxacin norflorcin and nalidixic acid, affect cell viability of resting chondrocytes much more seriously than growth plate chondrocytes mixture (containing all the hypertrophic, proliferative and some resting chondrocytes released from growth plate). Our findings indicate that the cell viabilities obtained after treatment for resting and growth plate cells were 83% and 93% respectively. Moreover, for drug concentration of 1 μg/ml (serum concentration), all quinolones tested showed significant inhibition on resting chondrocytes viability while only minimal effects on growth plate chondrocytes were detected. Chondrocytes released from these two different cartilage regions showed different responses to ofloxacin. Among the four quinolones tested in

this study, ofloxacin appears to be the most potent cytotoxic drug for resting chondrocytes but is the least potent one for growth plate chondrocytes.

At concentrations less than or equal to 1 μg/ml, no significant effect of quinolones on chondrocytes was observed. All quinolones (except ofloxacin) tested in this study showed a general inhibition on thymidine uptake in both resting and growth plate chondrocytes at higher concentrations. The effect appears to be more potent in resting chondrocytes than growth plate chondrocytes. At the concentration of 100 μg/ml, the maximum inhibition on thymidine uptake was 94% in resting chondrocytes while it was only 57% in growth plate chondrocytes. Although such high concentration of quinolones may not appear to be physiological, high accumulation levels have been reported in animals. For rabbits receiving a dose of 200 mg/kg of quinolone, the maximum serum and articular cartilage concentration of norfloxacin were 51.2 μg/ml and 46.3 μg/g respectively and those concentrations on nalidixic acid used in the treatments were even higher (Machida et al., 1990). Recently, Hayem and his co-workers (1994) demonstrated the local concentration of pefloxacin, a new type of fluroquinolone, in cartilage is 91.54 μg/g. Thus, the inhibition effect of quinolones on cell division in high concentrations may occur in clinical treatments.

Sulfate incorporation rate reflects matrix proteoglycan production activity. For proteoglycan synthesis activities, although nalidixic acid showed no effect on both resting and growth plate chondrocytes mixture, the effect of other quinolones are irregular in resting when compared with growth plate chondrocytes. In growth plate chondrocytes, all other quinolones perform a suppressive effect on sulfate incorporation. Norflorcin is the most potent inhibitor. Similar inhibitory effect of ofloxacin on sulfate uptake in rat articular cartilage was reported by Kato and Onoder in 1988. On the other hand, nalidixic acid demonstrated no effect while ciproxin and ofloxacin showed bi-phasic effect on sulfate incorporation in resting chondrocytes. Stimulatory response was even detected with norflorcin in 0.1 μg/ml, which showed the opposite response in growth plate chondrocytes.

All quinolones tested in this study can suppress activity of alkaline phosphatase, a chondrocyte differentiation marker, in growth plate chondrocytes. It indicates that quinolones may inhibit chondrocyte differentiation and in consequence hinder the development of growth plate thus suppressing bone growth.

The pathological effects of quinolones on resting zone and growing zone cartilage are different. In resting chondrocytes, the primary effect is direct cell killing. In the growth plate chondrocyte mixture which contains resting, proliferative and hypertrophic chondrocytes, the major pathology of quinolones is the prevention of chondrocyte differentiation. These observations provide us with further evidence for the hypothesis that porcine chondrocytes at various maturation stages have different phenotypic characteristics and respond differently in the presence of different modulators. It would be of clinical interest to test whether this finding can also be observed in patients, especially in children.

In conclusion, by using *in vitro* and *in situ* porcine costal growth plate models established in this study, we have successfully identified and separated various differentiative chondrocyte subpopulations. In addition, their phenotypic characteristics, such as basic biochemistry, glycoconjugate expression, calcium metabolism, electrophysiology and drug responses have been elucidated. The major phenotypic characteristics of porcine costal growth plate chondrocytes at various stage of differentiation investigated in this study is summered in Table 7.1. These findings confirmed our hypothesis that cartilage cells at various differentiation stages have different metabolic systems. The cells also respond differently to various hormones and stimulators. Therefore, the maturation stage should be clearly defined in all future investigations involving growth plate chondrocytes and the period of using a mixed population of chondrocytes for biochemical studies should be over.

Table 7.1 Summary of the Major Phenotypic Characteristics of Porcine Costal Growth Plate Chondrocytes at Various Stage of Differentiation Investigated in this Study.

	Resting Chondrocytes	**Proliferative Chondrocytes**	**Hypertrophic Chondrocytes**
Mean cell diameter (μm)	12.5	13.2	20.1
ALP ($\mu M/10^6$cells/hr)	0.3	4.5	5
Thymidine (f mol/10^6cells/hr)	75	275	25
Sulphate Incorporation (n mol/10^6cells/hr)	1	5	4
Subcellular[Ca^{2+} distribution	Nuclear region	Nuclear region / Submembrane region	Submembrane region
Stimulation of $[Ca^{2+}]_i$ by TGF-β_1	Yes	Yes/ No	No
Stimulation of $[Ca^{2+}]_i$ by TGF-α	Yes	Yes	Yes
Stimulation of $[Ca^{2+}]_i$ by PTH	No	No	No
Binding of WGA to membrane	Yes	No	No
Binding of DBA to membrane	Yes	No	Yes
Total K^+ current	Low	Low	High
K^+ current / unit surface area	Low	High	Low
Ca^{2+}-dependent K^+ current (%)	60	40	40
Ca^{2+}-independent K^+ current (%)	40	60	60
Viability to quinolones	Low	High	High
Differentiation inhibition by quinolones	Low	High	High

Potential Clinical Significance and Contribution from Results Obtained in this Study:

The growth plate is the specialized region in the physeal cartilage of long bone where proliferation of chondrocytes results in increase in longitudinal length and skeletal growth. Damage to the growth plate is permanent and irreversible. Children so affected, either as a result of trauma or infection, have severe and permanent joint deformities. The affected limb is shorter and deformed. Complicated major surgical reconstruction is often necessary to restore function in these limbs and the results are generally poor. There is no known medical or surgical method to effectively reverse damage to the growth plate.

Transplantation of biosynthetic growth plate is a new direction to overcome such skeletal growth defect. This area of basic research is far from being mature not only in Hong Kong, but for other countries as well. Our research group is ready to take up the challenge since we have already successfully separated and identified the resting, proliferative and hypertrophic chondrocyte subpopulations from pig growth plate. The proliferative chondrocytes will be cultured on collagen gel. Such biosynthetic growth plate will then be transplanted to a growth plate defected animal model. The growth plate reconstruction will be monitored. The parameters to be studied include morphological changes under light and electron microscopy, quality and quantity of matrix production and expression of differentiative markers. The effect of growth factors, such as TGF-β, on promotion of growth plate regeneration will also be investigated.

The establishment of biosynthetic growth plate transplantation system is also expected to offer solutions to other important and difficult orthopaedic problems such as repairing of articular surface in the degenerative joint diseases such as osteoarthritis, in which as many as 100,000 of total keen replacement are required to performed each year in the USA (Praemer 1992). For patients of metatropic dwarfism, growth plate transplantation seem to be the only means to restore them to

normality. Although callotasis has been used to treat achondroplastic dwarf, growth plate transplantation provide alternative treatment.

In the present study, we used pig as a model to produce subpopulations of chondrocytes. As far as xenotransplantion is concerned, pig is an ideal donor for human transplantation. Most recently, Tearle et al. (1996) found that knocking out the α(1,3)-galactosyltransferase gene encoding the human against pig xenoantigen, mainly galactose α(1,3)-galactose epitope on glycolipids and glycoproteins, provides a better chance for organ xenografts. The organs or cells produced from these transgenic pigs offer an ideal source for human. Whether chondrocytes from transgenic pigs can be employed in this aspect will also be of clinical importance.

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